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## Neural induction and differentiation of stem cells using the developmental gene PAX7

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**NEURAL INDUCTION AND  
DIFFERENTIATION OF STEM CELLS  
USING THE DEVELOPMENTAL GENE**

***PAX7***

By

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Supervised by:

**Dr. Mel Ziman & Dr. Meghan Thomas**



A thesis submitted in partial fulfilment of the  
requirement for the award of

**Bachelor of Science, Honours (Human Biology)**

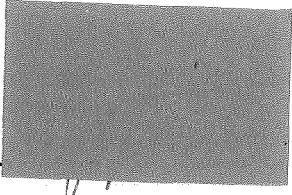
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## ABSTRACT

Neurodegenerative disorders are conditions that mainly affect the brain and the central nervous system (CNS). Each disease type is characterised by loss of function which results from death of a particular region. Interestingly enough, most of the time disease pathology is due to loss of specific cell types in the CNS. These pathological conditions have both high economic costs and social implications for society. To treat such conditions, either the lost cells must be replaced or the cells surrounding the damaged tissue must be induced to undergo repair to replace the lost cells. The former looks more achievable whereas the latter is problematic as the CNS has limited ability to regenerate. For cell replacement therapies, stem cells hold much promise in particular bone marrow stromal stem cells (BMSSCs).

Bone marrow stromal stem cells are phenomenal in terms of their plasticity, their ability to renew themselves and their availability. Just about every possible use of BMSSCs has been proposed from diverse fields, but little success has been achieved in same fields. BMSSCs have been used successfully for bone marrow transplants and to regenerate whole organs for cell replacement therapies. However current research has focused on differentiation of BMSSCs into neural tissue for cell replacement therapies for neurodegenerative disorders has had little success.

The initial approach was to optimise conditions for the growth and differentiation of BMSSCs. Once optimal conditions were characterised, experiments to induce differentiation of BMSSCs only produced a mixture of neuronal cells. Ideally for cell replacement therapy, only a single type of cell lost in the disease process needs to be replaced.

Pax7 is a key transcription factor that drives neural differentiation and migration of neural cells during development. It is also thought to determine neural

differentiation in the tectum, where it specifically drives the fate of precursor cells towards a neural lineage. Upregulation of the transcription factor Pax7 in stem cells may induce these stem cells to differentiate towards neural differentiation. Since Pax7 specifies neural cell fate and not any other cell type in the CNS, transfected cells should differentiate into neurons similar to those found in the tectum. It is hypothesised that transfecting stem cells with Pax7 would create a novel method of differentiating stem cells into a homogeneous population of neurons. The differentiated cells could then be used as cellular material for cell replacement therapies.

In this project a variety of stem cells, P19 cells, NIH3T3 cells and BMSSCs were transfected with *Pax7* and assessed for neural differentiation. P19 and NIH3T3 transfected cells clearly showed differentiation along a neurogenic lineage, specifically towards the neurons of midbrain. Sadly, the BMSSCs did not survive the transfection process and further optimisation of conditions is required to achieve neural differentiation of BMSSCs.

**Key words:** bone marrow stromal stem cells, differentiation and cell replacement therapy.

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### **List of abbreviations used**

BMSSCs	Bone Marrow Stromal Stem Cells
cDNA	complimentary Deoxyribonucleic acid
CNS	Central Nervous System
CRT	Cell Replacement Therapy
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate
EGF	Epidermal Growth Factor
ESC	Embryonic Stem Cells
FACS	Fluorescence Activated Cell Sorting
GFAP	Glial Fibrillary Acidic Protein
hFGF	human Fibroblast Growth Factor
HTH	Helix Turn Helix
LB broth	Luria-Bertani broth
NCAM	Neural Cell Adhesion Molecule
NSC	Neural Stem Cells
<i>Pax</i>	Paired box gene
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PSA	Polysialic acid
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TBS	Tris buffered saline
TXTBS	Triton X-100 Tris buffered saline

# **CHAPTER 1**

## **INTRODUCTION**



# **NEURAL INDUCTION AND DIFFERENTIATION OF STEM CELLS USING THE DEVELOPMENTAL GENE PAX7**

## **1.1 Hypothesis**

*Pax7* induces stem cells to differentiate into a homogeneous population of neurons *in vitro*.

## **1.2 Aims**

- 1.2.1 To stably transfect P19, NIH3T3 and Bone Marrow Stromal Stem Cells (BMSSCs) with a full length *Pax7* cDNA transcript (*Pax7d*).
- 1.2.2 To analyse neuronal differentiation of *Pax7*<sup>+</sup> stem cells.
- 1.2.3 To compare neural differentiation of three different *Pax7*<sup>+</sup> cell lines (P19, NIH3T3 and BMSSC).

## **1.3 Background**

Most neurodegenerative disorders are due to loss of a specific neural cell type in the central nervous system (CNS). Attempts to repair the damaged CNS have seen the development of cell replacement therapies. These therapies attempt to use embryonic stem cells or neural stem cells to replace neurons lost in injury or neurodegenerative disorders (Snyder and Olanow, 2005). However current cell replacement therapies utilise embryonic or foetal neural stem cells which are undifferentiated and are not fully characterised. Recently a lot of effort has been made to differentiate neural and embryonic stem cells *in vitro* which can then be utilised in cell replacement therapies. While these cells appear promising, there are limitations due to the source of the cells, cell plasticity, tissue compatibility and ethical implications. In an attempt to overcome these problems, an alternative source of cells is being developed, namely Bone Marrow Stromal Stem Cells (BMSSCs) that can be derived from the patients own bone marrow (Holden and

Vogel, 2002; Moore and Quesenberry, 2003). BMSSCs are easily accessible, have the ability to differentiate into various cell types, are tissue compatible and have few ethical considerations. Recent studies have shown that it is possible to differentiate BMSSCs into neurons (Sanchez-Ramos et al., 2000; Woodbury et al., 2000) but the differentiated stem cells are a mixture of different neuronal cell types (Azizi et al., 1998; Sanchez-Ramos et al., 2000; Woodbury et al., 2000). It is anticipated that in many cell replacement scenarios, it will be important to achieve a homogeneous population of the specific neuron cell type lost to disease or injury so that the function of the CNS can be restored.

Recently our laboratory has demonstrated that *Pax7* transfected into the P19 stem cell line was able to induce more than 95% neural differentiation (Thomas et al., 2004; Ziman et al., 2001). Moreover the differentiated cells were of one specific neuron cell type when characterised by specific neural markers (Thomas et al., 2004). *Pax7* encodes a transcription factor that is crucial for midbrain neural specification during development. Therefore *Pax7* may successfully induce stem cell differentiation along a neurogenic lineage *in vitro*.

#### **1.4 Significance**

In this project we stably transfected various stem cells with the gene encoding the transcription factor *Pax7*, and assessed differentiation of stem cells into a homogenous population of midbrain specific neurons. A variety of stem cells were transfected and the transfected stem cells were fully characterised using general neuronal markers as well as specific markers for midbrain neurons. This study also address the issue of achieving a homogeneous population of neurons derived from BMSSCs which other studies have failed to achieve. The outcome includes

the differentiation of stem cells into a homogeneous population of neurons that are specific to the midbrain. These experiments also provide the first step towards achieving a population *in vitro* differentiated cells that can be used for cell replacement therapies for neurodegenerative disorders and brain injuries associated with the midbrain.

## **CHAPTER 2**

### **BACKGROUND TO THE STUDY**

## **2.1 Neurodegenerative disorders**

### **2.1.1 The problem**

Neurodegenerative disorders are variable in their aetiology (Table 2.1) and often the causes are not clearly established. It is thought that both genetic and environmental factors contribute towards the outcome of the disease (Bertram and Tanzi, 2005). A common characteristic of many neurodegenerative disorders is the partial loss of neuronal function during disease onset followed often by neuronal death and complete functional loss in the final stages of the disease. Interestingly, most neurodegenerative disorders are characterised by loss of a single neural cell type in the CNS. For example Parkinson's disease (PD), which is of particular interest to this project, results from the loss of neurons of the midbrain. PD is characterised by the specific loss of dopaminergic neurons in the midbrain, substantia nigra which controls motor function. The onset of PD is clinically characterised by partial tremors of the hands, arms, legs and jaws. There is also stiffness of limbs and postural instability (Schapira, 1999). As the disease progresses more dopaminergic neurons are lost and the clinical symptoms become more pronounced and drug dependency increases (Schapira, 1999).

**Table2.1** Summary of neurodegenerative disorders that result from the loss of a single neuronal type in the CNS and the characteristics of replacement cells (Barker et al., 2003).

Disorder	Cause of neurodegenerative disorder	Target replacement cells
Parkinson’s disease	Loss of dopaminergic neurons	Nigrostriatal dopamine neurons
Multiple sclerosis	Oligodendrocyte in CNS	CNS oligodendrocyte
	Schwann cells in PNS	Schwann cells in PNS
Amyotrophic lateral sclerosis	Loss of alpha motor neurons	Replacement of alpha neurons
Huntington’s disease	Loss of neurons in basal ganglia and cerebral cortex	Replacement of basal ganglia and cerebral cortex specific neurons
Alzheimer’s disease	Deposition of amyloid causing death of neuronal tissue	Diffused neuronal replacement
Frontotemporal dementia	Loss of neurons in frontal and temporal lobes of the brain	Replacement of frontal and temporal lobe neurons

A second important characteristic of many neurodegenerative disorders is the accumulation of protein in the neural tissue which results in cell death (Bilen and Bonini, 2005). Although the accumulation of protein in PD is not well characterised, in other neurodegenerative diseases, such as Alzheimer’s disease, the disease symptoms are due to the accumulation of beta amyloid in the neural tissue (Verdile et al., 2004). Other form of brain disorders such as damage due to physical or chemical induced injury can affect specific brain regions. Damaged neurons do not self regenerate, so the lost neurons need to be replaced to restore the function of the particular brain region.

**2.1.2    Current treatment**

Most of the current treatments for neurodegenerative disorders are aimed at relieving the symptoms rather than repairing the damaged tissue and curing the disease (Armstrong and Barker, 2001). For example during the onset of PD, the symptoms are mild and the base line treatment is dopamine-replacement. Levodopa is

particularly effective during early stages, however as the disease progresses, dopamine replacement does not provide any therapeutic benefits (Schapira, 1999).

Neurodegenerative disorders are more common in elderly patients and there are serious complications associated with polypharmacy in elderly patients. Long term over dependency on drugs results in less therapeutical benefits and more side effects. For these and other reasons, most research is now aimed at stem cell replacement therapies.

Two definitive ways to treat neurodegenerative disorders are:

1. Stop or reduce the protein accumulation that results in cell death or,
2. Replace the cells that have been lost.

Replacing the lost neural cells seems to be a more feasible option, as cell death has already occurred when the disease is diagnosed.

### **2.1.3 Cell replacement therapy**

Cell replacement therapy involves transplanting embryonic stem cells or adult stem cells at the site of injury to facilitate regeneration of the damaged tissue, and has generated much interest as a means of treating neurodegenerative disorders. For successful transplant therapy, the lost cells should have modulatory action that does not require complex and precise patterns of connectivity (Bjorklund and Lindvall, 2000). Cell replacement therapy is therefore a feasible solution for neurodegenerative disorders that result from loss of a single type of neuron in the CNS, such as PD (Barker et al., 2003).

There are different approaches towards treating neurodegenerative disorders using cell replacement therapies. The first approach is to stimulate already existing neural stem cells in the CNS to proliferate and differentiate. This treatment is non-invasive

and overcomes the need for immunosuppression (Falk and Frisen, 2005). The second approach, that of neurodegenerative cell replacement therapies was initially trialled using undifferentiated stem cells transplanted into the damaged CNS. It was assumed that the undifferentiated transplanted cells would differentiate into appropriate neural types depending on the micro-environment of the CNS. The studies by Azizi and colleagues (1998) clearly demonstrated that rat mesenchymal stem cells, isolated from bone marrow and infused into the rat brain, successfully engrafted, migrated and survived (Azizi et al., 1998), albeit in very low numbers. However very few cells differentiated into neurons.

A more recent approach has been to differentiate cells *in vitro* and then transplant them into the CNS to restore the functional deficiencies caused by neurodegenerative disorders. Through those approaches, a variety of neurological disorders can be treated but the most progress has been made in treating PD.

Promising but variable results have also been obtained by transplanting aborted foetal tissue from the embryonic midbrain (Bjorklund and Lindvall, 2000). However there have been a number of setbacks with this approach: several foetuses are required to obtain sufficient cells, plus there are ethical concerns over the use of foetal tissue (Falk and Frisen, 2005). From the results of their experiment, though it appears that transplanting undifferentiated neuroblast to replace those that have degenerated in PD is a feasible approach to restore functional integrity of the nigrostriatal dopamine system, for patients that do not respond well to medical and pharmacological therapy (Olanow et al., 1996).

Several studies have now been performed using embryonic stem cells, foetal neural stem cells as well as adult stem cells in animal models. There is great support



from these studies for stem cell therapies for neurodegenerative disorders especially PD (Kim et al., 2002; Studer, 1998)

#### **2.1.4 Stem cells from adult tissue**

In the mid 19<sup>th</sup> century, Cohnheim suggested that all adult stem cells reside in the bloodstream (Cohnheim, 1867). The hypothesis has been an issue of immense discussion and research since then. Recent observations have shown that small, stem-like cells with considerable plasticity are found in a variety of adult tissues including muscles, fat, liver, synovial membrane and the brain (Ferrari et al., 1998; Mezey, 2000; Petersen et al., 1999). Those cells self renew and also differentiate during tissue repair. This has validated Cohnheim's hypothesis. It is clear that stem cells found in most tissues are replenished to a small extent by stem cells found in the marrow (Prockop et al., 2003).

Unlike other cells in the body, adult stem cells are characterised by two distinct properties: self renewal and the ability to differentiate into mature cells (McKay, 1997; Weissman, 2000). When a stem cell divides, it undergoes asymmetric cell division resulting in one stem cell and one differentiated mature cell (Majka et al., 2005), thus reserve populations of stem cells are maintained for further cell division.

Several types of stem cells exist; totipotent stem cells from the zygote, from which both embryo and placenta are produced; pluripotent stem cells which give rise to only the embryo, and multipotent stem cells which give rise to the three germ layers: endoderm, mesoderm and ectoderm. Finally stem cells within adult tissues are generally committed stem cells which give rise to cells of a particular tissue (Lemoli et al., 2005). From one cell at fertilisation to billions of cells in an adult, cells undergo continuous differentiation and replacement. But not all cells in the body differentiate;

some remain as undifferentiated primitive precursor cells and upon damage to the tissue, help in the regeneration of the tissue (Johansson et al., 1999; Lemoli et al., 2005).

The major cell types in the CNS are neurons, astrocytes and oligodendrocytes. They are derived during development from neuronal stem cells formed in the regenerative zone along the entire neuraxis (Gage, 2000; McKay, 1997). Since the previous decade, the adult mammalian neural system was considered to be non-renewable tissue (McKay, 1997), but this principle has been challenged by studies which have shown that neuronal progenitor cells found in the CNS are capable of cell division (Falk and Frisen, 2005).

The first evidence of neural stem cells in the brain came from the *in vitro* culturing of adult brain cells. Cells were cultured in the presence of growth factors and a small number of cells proliferated to form neurospheres. These cells were then able to differentiate into neurons, astrocytes and oligodendrocyte, thus showing characteristic of true stem cells (Reynolds and Weiss, 1996; Richards et al., 1992). In addition, the observed changes in neurons in the adult brain of songbirds, important for the annual change of song patterns, provided further evidence of addition of neurons in the mature brain (Nottebohm, 2004). Newly generated neurons in the adult brain are derived from neural stem cells formed in the sub-ventricular zone and hippocampus (Gage, 2000).

Although the adult human brain demonstrates regeneration in specific areas, there is a limit to the extent to which the CNS will regenerate. It is believed that this is due to lack of sufficient endogenous factors to activate endogenous neural stem cells in sufficient amounts to repair the damaged CNS (Peterson, 2002). Recent experiments suggest that BMSSCs can be induced to differentiate into neural cells (Grove et al.,

2004). Thus BMSSCs could be used for neural cell transplant therapies if BMSSCs could be induced to differentiate into a specific neural cell type.

#### **2.1.5 Limitations of cell replacement therapies**

Several studies have shown that transplanted stem cells do not necessarily differentiate into a required specific neural cell type. For functional benefits, transplanted cell should be able to integrate into the mature brain and establish appropriate connections (Falk and Frisen, 2005). Cell replacement therapies should therefore be used to replace cells that do not require complex connections and have relatively simple functions. The other challenge for successful cell replacement therapy is to control cell differentiation to produce only one specific cell type. The other challenge is to control proliferation once the cells are transplanted in the CNS; otherwise tumour formation results (Falk and Frisen, 2005). Once the differentiated cells have been transplanted in the CNS, they should lose their proliferative properties and become tissue committed cells which cannot proliferate further.

Though the stem cell isolation methods are well established, a major setback is that little is known about the safety of transplanting stem cells subjected to current isolation methods. Generally these cells are labelled with antibodies and conjugated with fluorescent dye or magnetic particles and sorted using fluorescence activated cell sorting (FACS) or a magnetic field. These methods tend to employ chemicals and dyes such as Hoechst 33342 and Rhodamin 123 (Goodell et al., 1996; Ratajczak et al., 1998). The effects of these dyes on the stem cells are not yet established and could change the primitive fate of the stem cells. This could limit the ability of stem cells to differentiate along a particular lineage and may be a reason why little success has been achieved in differentiating stem cells into homogenous populations of neurons.

BMSSCs can also be sorted in accordance with their property of adherence to plastic (Rando and Blau, 1994). BMSSCs isolated by adherence to plastic are not actually homogeneous but the cells become homogeneous in appearance when grown in culture for longer and they lose their haematopoietic properties (Ono et al., 2003).

### **2.1.6 The ideal cells**

As previously discussed (1.3), a major limitation of cell replacement therapy is sourcing of the cells. The cells predominantly used are embryonic stem cells and neural stem cells. However the ideal replacement cells for successful cell replacement therapy should meet six conditions:

1. Lots of cells should be available for harvesting with no risk of depletion.
2. The cells should be compatible with indefinite expansion *in vitro* without loss of plasticity.
3. The harvested cells should be able to differentiate into a homogeneous population of cells.
4. The cells should possess plasticity or be able to undergo trans-differentiation into specific mature neurons able to integrate into the mature brain upon transplantation.
5. There should be no risk of cell rejection after transplantation.
6. There should be no risk of cell proliferation and formation of tumours.

BMSSCs are ideal cells that would meet all of the above criteria and overcome major problems that currently face cell replacement therapy. BMSSCs replenish themselves and can be either harvested from bone marrow or blood, thus are easier to access than neural stem cells and embryonic stem cells. Transgenic plasticity is a key

property of bone marrow stem cells; they are able to differentiate into cells that are phenotypically unrelated to the tissue of origin (Bianco et al., 2001). Lastly, since the cells are isolated from the patients own bone marrow, they do not pose a threat of cell rejection and thus no immunosuppression would be required to graft the cells into the CNS.

## 2.2 Bone marrow stromal stem cells (BMSSCs)

### 2.2.1 BMSSC plasticity

Bone marrow stromal stem cells normally contribute to the regeneration of mesenchymal tissue such as bone, cartilage, ligaments, tendons and muscles (Bruder et al., 1997; Ferrari et al., 1998; Petersen et al., 1999). Studies by Woodbury et al, (2000) and Sanchez-Ramos et al, (2000) have shown that rat bone marrow can be induced to differentiate into neurons and astrocytes *in vitro* and BMSSCs when transplanted into the damaged CNS, have the potential to migrate and differentiate into neuronal tissue in animal models (Sanchez-Ramos et al., 2000; Woodbury et al., 2000).

Interestingly BMSSCs transplanted into the brain have a tendency to differentiate into neurons whereas BMSSCs transplanted into the spinal cord have a tendency to differentiate into astrocytes (Shihabuddin et al., 2000). This is an important finding as it indicates that the fate of transplanted BMSSCs is directed by the micro-environment of the host tissue rather than the pre-programmed fate of the cells themselves (Spradling et al., 2001; Watt and Hogan, 2000).

Results from our lab suggest that *Pax7* is a key micro-environmental factor in regionalisation and specification of neurons of the superior colliculus (or the tectum in lower vertebrate). Thus transfecting BMSSCs or any stem cell with *Pax7* could create a similar micro-environment to that found *in vivo* and induce differentiation of stem cells into neurons specific to the superior colliculus.

### **2.2.2 Ethical considerations**

Embryonic stem cells, which are derived from the inner cell mass of the embryo, are the most totipotent cells and are capable of differentiating into any cell of the body. The recent controversy surrounding the procurement of embryonic stem cells has divided people into supporters of therapeutic cloning and non-supporters of therapeutic cloning. Recent legislation permits the use of these stem cells for therapeutic cloning. However ethical considerations remain a limiting factor.

There are few, if any, such ethical considerations when harvesting BMSSCs. The harvesting of BMSSCs is done by withdrawing bone marrow from an adult, typically patients themselves. It does not involve any long term/irreversible procedure thus overcoming the ethical considerations associated with the use of embryonic stem cells.

### **2.2.3 Supply and availability**

The greatest advantage of BMSSCs is the fact that they are readily available in the blood and in the bone marrow of the adult patient. The cells can be isolated from blood by injecting a growth factor, colony stimulating factor into the blood stream of the patient for five days which enhances proliferation of these cells. Haematopoietic stem cells are then collected through apheresis (Cutler and Antin, 2001).

An alternative way of harvesting BMSSC is by inserting a needle into the shaft of the femur or tibia and withdrawing bone marrow (Sanchez-Ramos et al., 2000).

BMSSCs have also been harvested from cord blood. The first cord blood bank was established in 1995 in Australia, allowing patients access to stem cells from their own cord blood. The practise of cord blood collection is fairly recent and the storage procedures are complex and expensive (Australian National Pathology Accreditation Advisory Council., 1999). This places a major limitation on the use of cord blood derived stem cells relative to the more accessible BMSSCs harvested from bone marrow.

#### **2.2.4 The risk of cell rejection**

When using BMSSCs cell rejection would not arise because the cells are from the persons own bone marrow. According to the Bone Marrow Donor Institute of Australia, the only limitation associated with use of patient's own BMSSCs is that, if there is a genetic defect in BMSSCs since birth, then that defect would also be present in the BMSSCs and may affect their ability to differentiate into different lineages. This is an important issue to address in neurodegenerative disorders like familial Huntington's disease, where a gene defect cause the disease, and BMSSCs would have the same defect as the brain cells. BMSSCs are therefore not a solution where a germline mutation is present.

In this project I will investigate the ability of the developmental gene *Pax7* to differentiate stem cells including BMSSCs to a homogeneous population of neural progenitor cells for later use in cell replacement therapy to repair damage to the superior colliculus. *Pax7* is just one of several *Pax* genes that could be used for differentiation of region specific neural cells for cell replacement therapy.



## **CHAPTER 3**

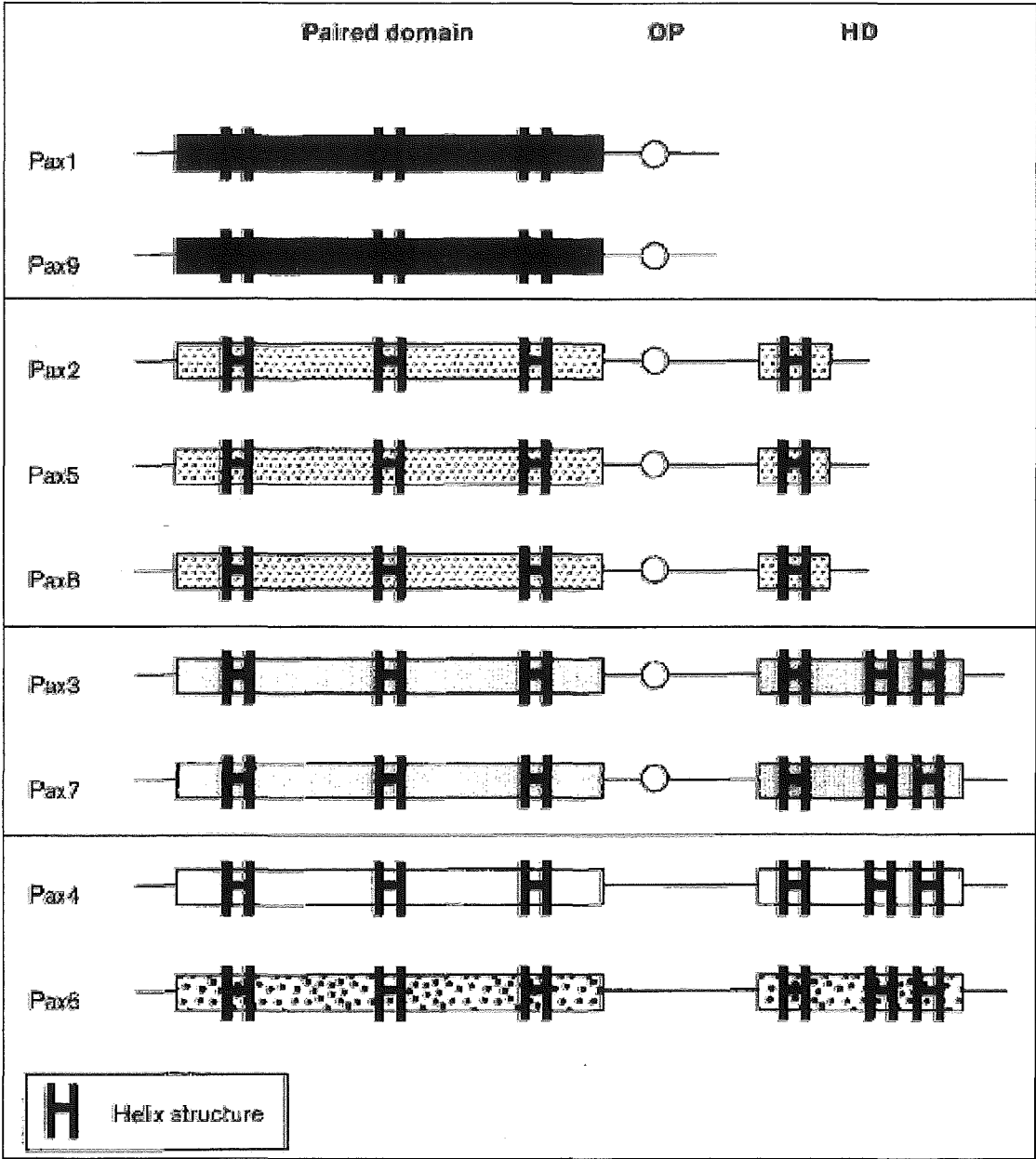
### **ROLE OF *PAX7***

### **3.1 Role of Pax genes**

*Pax7* is one of several *Pax* genes which encode a family of transcription factors that are critical during development and organogenesis (Dahl et al., 1997; Mansouri et al., 1996a). In vertebrates, *Pax* genes have been conserved over millions of years (Jostes et al., 1990) and play very important roles in CNS, muscle, thymus, kidney and pancreatic development during embryogenesis.

The expression of *Pax* genes in the CNS are region specific, contributing to regionalisation of the brain during embryogenesis (Stoykova and Gruss, 1994; Ziman and Kay, 1998). *Pax* expression is up-regulated during embryogenesis but expression becomes restricted to specific cells as the embryo moves towards adulthood.

The *Pax* gene family has nine *Pax* members which encode Pax proteins that have a paired DNA binding domain. There are four Pax protein subgroups and their division into subgroups is based on their relative developmental function and degree of amino acid conservation (Gruss and Walther, 1992; Jostes et al., 1990; Mansouri et al., 1996a) refer to figure 3.1.



**Figure 3.1.** Classification showing four subgroups of Pax genes, adapted from (Mansouri et al., 1996a)

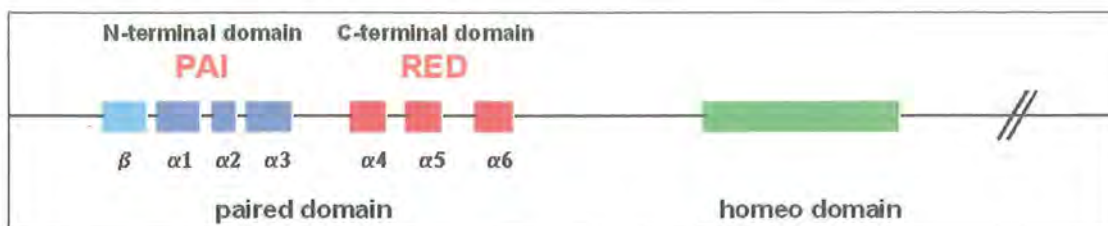
Pax proteins primarily bind to an enhancer DNA sequence in the promoter region of target genes and regulate expression of the target gene, whose functional products control developmental pathways (Kawakami et al., 1997). The importance of *Pax* genes has been shown in mutant mice which do not survive to birth and develop severe deformalities (Chi and Epstein, 2002). Mutations in *Pax* genes cause failure of whole organs to develop as demonstrated by *Pax3* mutant mice which exhibit neural

tube defects and multiple neural crest deficiencies (Lang et al., 2000). Moreover, *Pax6* controls the formation of the eye in vertebrates and invertebrates. *Pax6* heterozygous mice have a small eye while homozygous mutant mice lack eyes. This not only proves that *Pax6* is required to regulate downstream genes that form the eye but also indicates that the effects of *Pax6* are concentration specific (Halder et al., 1995; Quiring et al., 1994).

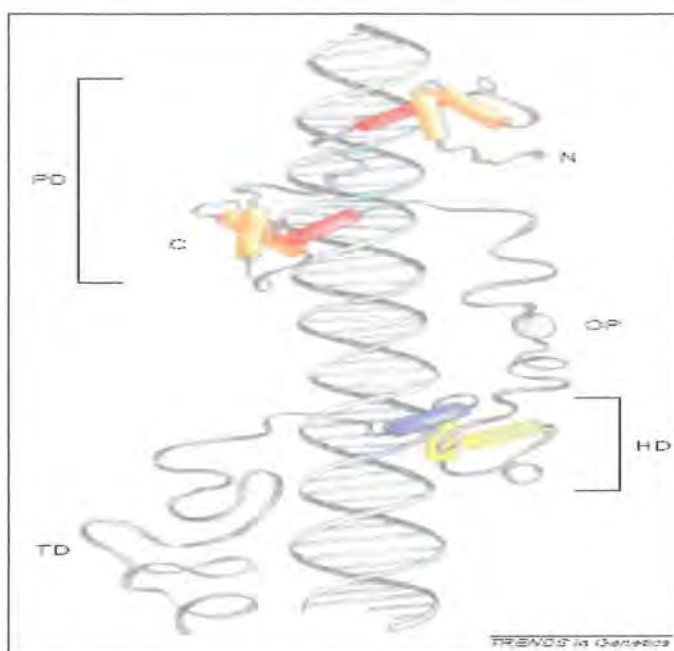
Most *Pax* genes are expressed as alternate transcripts with variations influencing the efficiency and specificity of binding by the encoded protein to the target DNA (Vogan and Gros, 1997). Thus Pax transcription factors exhibit wide functional diversity and play important roles spatially and temporally to achieve the complex configuration of the developing embryo.

### **3.2 The structure of Pax protein**

The Pax family of transcription factors have two DNA binding domains and a protein interaction domain; a paired domain, a homeodomain and a transactivation domain respectively (Ziman and Kay, 1998). The paired domain consists of six  $\alpha$ -helices grouped into two helix-turn-helix (HTH) motifs. The paired domain is 128 amino acids long and is located close to the N-terminal end of the protein (Jostes et al., 1990) and consists of two sub domains (PAI and RED) each consisting of three  $\alpha$ -helices (Bopp et al., 1986). Each sub domain may bind independently to target DNA sequences. Moreover, the homeodomain may bind independently to DNA or at site adjacent to those bound by the paired domain. Refer to figures 3.2 and 3.3.



**Figure 3.2.** The structure of the Pax protein: The paired domain consists of two sub-domains PAI and RED, each with three  $\alpha$ -helices (Bopp et al., 1986). The conserved octapeptide region is located between the paired domain and the homeodomain.



**Figure 3.3.** Representation of the structure of the Pax protein with the paired domain (PD), octapeptide motif (OP), homeodomain (HD) and transactivation domain (TD) indicated. All Pax proteins have a similar structure but vary in the presence of a complete or partial homeodomain and/or octapeptide (Chi and Epstein, 2002).

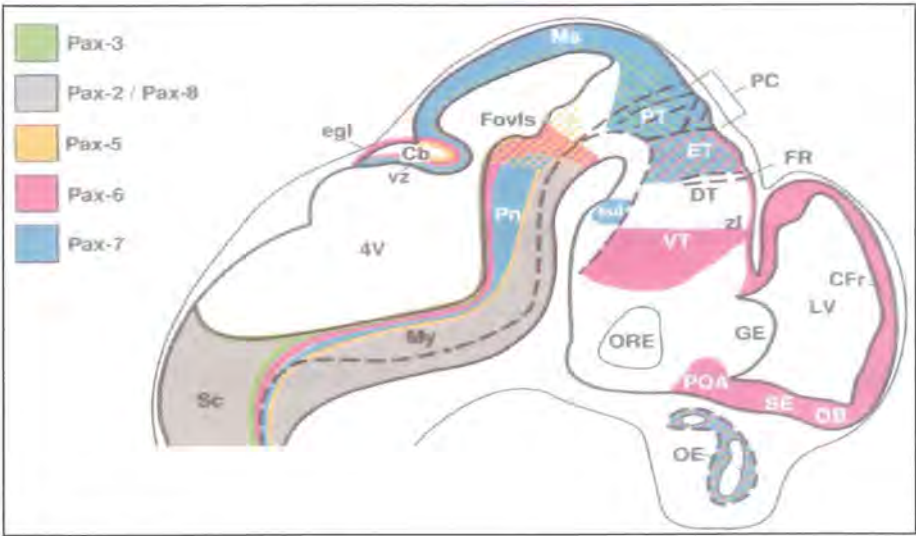
### 3.3 Expression patterns of Pax genes

Most of the *Pax* genes are expressed in the spinal cord apart from *Pax1*, *Pax4* and *Pax9* which are expressed in the vertebral column.

**Table3.1:** Expression of *Pax* genes in the CNS and their location. Adapted from (Underhill, 2000).

<i>Pax</i> gene	Location of expression
<i>Pax1</i>	Not expressed in CNS, expressed in developing vertebral column
<i>Pax2</i>	Midbrain-hindbrain boundary & optic stalk
<i>Pax3</i>	Mesencephalon
<i>Pax4</i>	Endocrine pancreas- not in the CNS
<i>Pax5</i>	Midbrain-hindbrain boundary
<i>Pax6</i>	Telencephalon
<i>Pax7</i>	Mesencephalon
<i>Pax8</i>	Midbrain-hindbrain boundary
<i>Pax9</i>	Not expressed in CNS, expressed in developing vertebral column

Though several *Pax* genes are expressed in the brain during development, it is the restricted expression of each *Pax* gene that helps to establish regional boundaries in the brain.



**Figure 3.4.** Diagrammatic representation summarising the expression of *Pax* genes within distinct boundaries in the embryonic brain. Adapted from (Stoykova and Gruss, 1994)

### 3.4 The function of Pax7 in the CNS

During embryogenesis, *Pax7* exhibits discrete expression patterns in the CNS, as well as in migrating neural crest cells, and condensing somites (Jostes et al., 1990). Mansouri and colleagues have shown that *Pax7* participates in development of neural-crest-derived cranio-facial structures and skeletal muscle satellite cells by using knock-out mice (Mansouri et al., 1996b; Seale et al., 2000). *Pax7* is expressed spatially and temporally in the CNS during early embryogenesis (day 8) through to postnatal development (Jostes et al., 1990) and in adults (Ziman et al., 1997).

Alternate splicing creates alternate transcripts of *Pax7*, which encode isoforms with different structures. Each isoform is likely to bind to a different set of target genes (Ziman and Kay, 1998). *Pax7* expresses four alternate transcripts; *Pax7a*, *Pax7b*, *Pax7c* and *Pax7d*, which differ by the presence or absence of a trinucleotide or a hexanucleotide in the paired box. This results in the respective encoded proteins having one or two extra amino acids included or excluded in the paired DNA binding domain.

It is the inclusion or exclusion of one, two or none amino acids in the paired domain that determines the specificity of *Pax7* isoforms for their target genes. Previous studies have shown that while the proportion of alternate transcripts is similar in brain and muscle, the more frequent combination of transcripts in brain are *Pax7b* and *Pax7d* while in the muscle the more common combination is *Pax7a* and *Pax7c* (Ziman and Kay, 1998). The transcripts expressed in the brain lack a hexanucleotide which suggests they may have a role in neurogenesis (Ziman and Kay, 1998). Therefore in this project the *Pax7d* isoform was utilised as it is the most abundant transcript and is found predominantly in neural cells (Ziman et al., 1997).

### 3.5 Why transfect stem cells with Pax7?

*Pax7* has been shown to participate in neurogenesis of the midbrain during development. *Pax7* is a transcription factor that binds and regulates the expression of downstream target genes which are likely to be involved in neural differentiation in the midbrain (Thomas et al., 2006; Thomas et al., 2004; White and Ziman, 2006). *Pax7* is expressed in the mesencephalic alar plate as early as day two during development and plays a role in tectum development (Jostes et al., 1990; Thomas et al., 2006; Thomas et al., 2004). In fact, *Pax7* may be regarded as a master control gene for specification of the tectum as it defines the identity of the dorsal alar plate and ectopic expression of *Pax7* in the forebrain induces formation of an ectopic tectal structure (Matsunaga et al., 2001; Nomura et al., 1998).

Furthermore *Pax7* is expressed in the majority of proliferative cells during early stages of tectal differentiation. However the expression pattern of *Pax7* in later stage tectal laminae is not homogeneous but rather graded which suggests its importance in establishing tectal polarity and laminar architecture (Thomas et al., 2006; Thomas et al., 2004). The protein is expressed in graded concentrations, high dorsally and low ventrally, low rostrally and high caudally (Thomas et al., 2006). Thus *Pax7* is expressed in distinct boundaries within the mesencephalon which helps to establish tectal regionalisation and polarity in the mesencephalon. It is the spatial and temporal expression of *Pax7* that regulates development of the tectum (Thompson et al, 2006. In press).

*Pax7* is continuously expressed in the retino-recipient precursor laminae of the tectum with relative changes in *Pax7* expression as differentiation occurs. Moreover *Pax7* appears to play a role in the specification of subtypes of neurons destined to occupy retino-recipient precursor laminae (Thomas et al., 2006).



Our previous research has shown that stem cells transfected with *Pax7* can be differentiated along a neurogenic lineage (Thomas et al., 2004; Ziman et al., 2001). Conclusive data showed that a P19 cell line stably transfected with *Pax7* resulted in more than 95% of the cells differentiating along the neurogenic lineage (Ziman et al., 2001).

Moreover, three days after injury to the tectum (or superior colliculus in higher vertebrates), there is up regulation of *Pax7* which presents a small window of opportunity for regeneration to occur (Thomas et al., 2006). In organisms capable of retinal regeneration, up regulation of *Pax6* has also been observed; cells enter the cell cycle and repopulate the damaged retinal cells (Chi and Epstein, 2002). The results of these studies suggest that there are endogenous mechanisms that may assist with regeneration of damaged tissue (Thomas et al., 2006; Yamamoto et al., 2001). Other studies have shown that adult neurogenesis can be stimulated by basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF) in an adult brain (Gould et al., 1999; Palmer et al., 1999).

Stem cells have also been shown to differentiate into neurons through growth factor induction *in vitro* (Joannides et al., 2003). The major set back with growth factor induced neuronal differentiation is that differentiated cells are not well characterised and they are more often a mixture of different neuronal cell types.

Given that *Pax7* is the key regulator for neural differentiation in the dorsal midbrain and is expressed in the damaged brain three days after injury and given that one of the key reasons for incomplete regeneration of the CNS after damage is due to lack of sufficient endogenous factors (Peterson, 2002), it is possible that there is insufficient *Pax7* to reach threshold values to cause neural proliferation, differentiation and repair in the adult midbrain. This study addresses the shortfall of

endogenous factors that drive neuronal differentiation by up regulating *Pax7* in stem cells that can be used for cell replacement therapies to replace lost neurons.

### **3.6 Implications of using *Pax7* in stem cells for cell replacement therapy**

The potential to generate a homogeneous population of a particular type of neuron would enhance cell replacement therapy for diseases caused by loss of a single type of neuron. The use of a transcription factor that is involved in specification of neurons during development and which is up regulated during an injury or a disease state, to differentiate stem cells along a neurogenic lineage, may induce a homogeneous cell population for cell replacement therapies (Rossi and Cattaneo, 2002).

## **CHAPTER 4**

### **METHODS AND MATERIALS**

#### 4.1 Cloning of Pax7d into pcDNA3.1D/V5-His-TOPO vector.

The vector used for cloning was pcDNA3.1D/V5-His-TOPO vector (Invitrogen) Appendix 1; it generates consistent high gene expression in mammalian cells compared to other commercially available vectors.

cDNA of *Pax7d* transcript (Genebank accession number: NM\_011039.1 Appendix 2) cloned into pcDNA 3.1/V5 HIS TOPO vector was kindly provided by Rob White (White and Ziman, 2006). Vectors were transfected into *E.Coli*, plated on Luria-Bertani (LB) agar plates containing 100 µg/ml of ampicillin, incubated overnight at 37°C. Individual colonies from the plates were selected and grown in 5ml of LB broth overnight at 37°C on a rocker. The pc DNA 3.1/V5-HIS-TOPO vector map is provided in Figure 4.1. The cloned *Pax7* sequence is also provided in appendix 2.

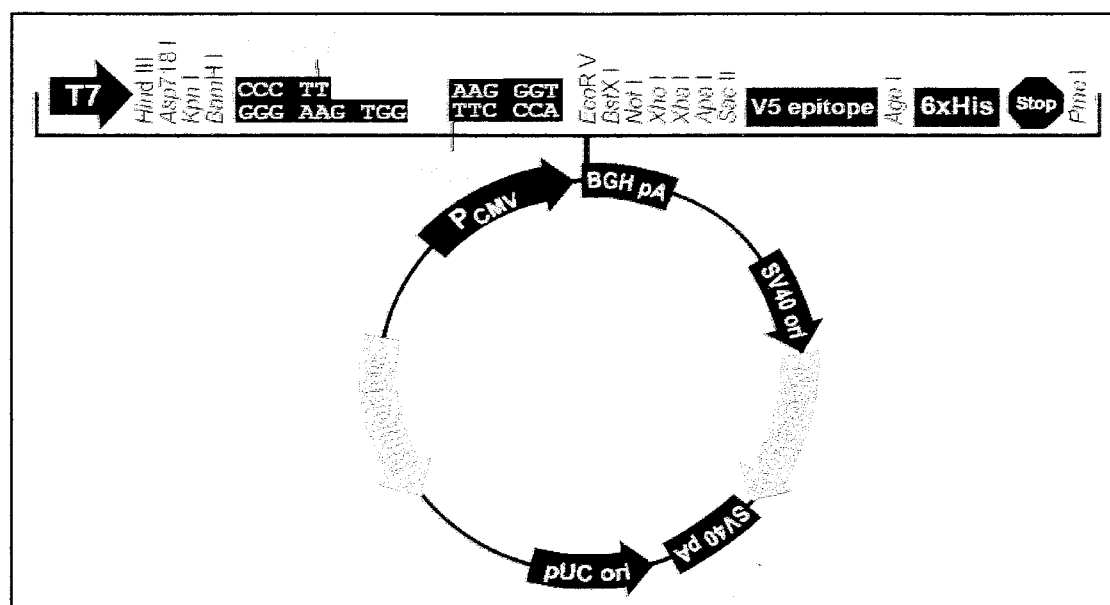


Figure 4.1. Graphical representation of the pcDNA 3.1/V5 HIS-TOPO vector. *Pax7d* was inserted between the two TOPO sites. The vector contains a V5 epitope tag and a His6 tag. The vector contains a neomycin resistant gene for selection of stable transfected colonies in mammalian cells.

Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. *E.Coli* containing LB broth was centrifuged, supernatant removed and the pellet resuspended in 250 µl of buffer P1. The cells were lysed in 250 µl alkaline solution buffer P2 for 5 minutes. The solution was neutralised using 350 µl of buffer N3, mixed gently and centrifuged at 13000rpm for 10 minutes. The supernatant was then applied to a silica membrane spin column, and the plasmid DNA was allowed to bind to the silica membrane. RNA and proteins were removed by centrifuging for 1 minute at 13000 rpm. The silica membrane was washed twice with buffer PB to remove any other impurities before DNA was eluted with 50 µl of sterile water.

Plasmid DNA (5 µl) was loaded with 3 µl of loading buffer onto a 1% agarose gel containing ethidium bromide 0.1 µl/ml and electrophoresed at a constant voltage of 105 V for 1 hour. A 1 kb ladder was utilised for relative size comparisons. The gel was visualised on a Gel Doc System (BioRad) and images analysed using the Quantity One software program (BioRad). DNA concentrations were calculated by spectrophotometry at a range of dilutions.

To confirm that *Pax7* was inserted into the vector in the correct orientation, the *Pax7d* insert region was amplified by PCR using the forward primer (F4) 5'-CACCGACTCTGGATTCGTCTCCAGCGTG-3', of which the first four bases are vector specific while the rest of the primer spans the 5' untranslated region of *Pax7*. The reverse primer, BGH reverse 5'-TAGAAGGCACAGTCGAGG-3', is complementary to the vector sequence at the 3' end of the *Pax7* insert. The melting temperatures were calculated using a web based primer analyser (Integrated DNA technologies,

<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx> ).

The PCR reaction was performed with QIATaq (QIAGEN, Australia) according to the manufacturer's specifications. Briefly, the PCR reaction contained 1x PCR buffer (15 mM MgCl<sub>2</sub>), 1x Q-solution, 10 µM forward primer, 10 µM reverse primer, 10 µM dNTPs, 0.5 units of Taq polymerase per reaction and 50 ng DNA in a volume of 20 µl. PCR cycle conditions were: Initial denaturation for 3 minutes at 94°C; then 30 cycles of denaturation for 1 minute at 94°C, annealing for 30 seconds at 50°C and 2 minutes extension at 72°C followed by a final extension for 10 minutes at 72°C.

PCR products (5 µl of sample with 3 µl of loading buffer) were electrophoresed on a 1% agarose gel containing ethidium bromide (0.1 µl/ml). The gel was run at a constant voltage of 105 V for 1 hour. The gel was visualised and the image was captured on a Gel Doc System (BioRad). The expected PCR product size was 1744 bp.

#### **4.2 Ethics**

This project involved use of rats to isolate bone marrow stromal stem cells. For this reason, ethics clearance was obtained from the Animal Ethics Committee at Edith Cowan University. All necessary protocols were followed so as to cause minimal pain to the animals. This project also involved genetic modification to cells and therefore necessary notification was made to the Institutional Biosafety Committee (IBS) for classification of the project as an exempt dealing in accordance with Gene Technology Act (2001) (IBC ref number: ED.4.2005).

#### **4.3 Bone marrow extraction**

Animal ethics clearance was obtained from Edith Cowan University Animal Ethics Committee, (approval number 06-A3) prior to purchasing and euthanizing the animal.

An 8 week old Sprague Dawley Rat (285 grams) was purchased from the Animal Resource Centre (Murdoch University, Perth). Upon arrival, the rat was euthanized with Lithabarb (Sodium Pentobarbitone) at a concentration of 15 mg/100 grams of body weight by intra-peritoneal injection (1ml injection with 20G needle). Death was confirmed by lack of breathing and no response after pinching the toes.

Using a surgical scalpel and a scissors, skin and muscles were cut to expose the femur and tibia, which were then physically detached from the body and cleaned of excess muscle and tendons. In a Petri dish, the ends of the femur and tibia were cut to expose the shaft of the bone. Bone marrow was extracted by inserting a 21G needle and flushing each bone with 5 ml of  $\alpha$ -MEM. The cell suspension was centrifuged at 500rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 1 ml culture medium. Resuspended cells were grown in T25 culture flasks in 5 ml of culture medium. Culture medium comprised of  $\alpha$ -MEM (Invitrogen), 15 % knockout serum (Invitrogen), and 2 mM Glutamax (Invitrogen).

#### **4.4 Culture BMSSCs, P19 cells and NIH3T3 cells**

BMSSCs isolated as described above, and a BMSSCs cell line (purchased from Tulane centre for gene therapy, New Orleans), were initially cultured in medium consisting of  $\alpha$ -MEM, knockout serum and Glutamax for one week. Medium was changed three times a week. The BMSSCs were then cultured in growth medium for three weeks to obtain a pure population of BMSSCs. Growth medium consisted of

42%  $\alpha$ -MEM (Invitrogen), 42% F12 (Invitrogen), 15% knockout serum (Invitrogen) or fetal calf serum (Invitrogen), 20 ng/ml FGF (Invitrogen), 20 ng/ml hEGF (Invitrogen) and 100 U/ml penicillin/streptomycin/Fungizone.

Two cell lines were also cultured alongside the BMSSCs for experimental comparisons. P19 embryonic carcinoma stem cells (passage 12, ATCC, catalogue number CRL-1825) and NIH3T3 embryonic fibroblast (passage 10, ATCC, catalogue number CRL-1658) were thawed from frozen stocks and resuspended in 1 ml of growth medium. Resuspended cells were transferred to T25 culture flasks and grown in 5 ml of growth medium at 37°C in 5% carbon dioxide. The growth medium was changed three times a week. P19 is an embryonic carcinoma stem cell line which has previously been shown to differentiate into neuronal or muscle cells. NIH3T3 is an embryonic mouse fibroblast stem cell line commonly used for DNA transfection studies. The BMSSC line is the preferred choice for cell transplant therapies as both P19 and NIH3T3 cell lines cause tumours, but are useful for development of *in vitro* stem cell models.

#### **4.5 Passaging the cells**

When cells reached 90-95% confluency, the growth medium was aspirated and discarded. Cells were washed with 2 ml of PBS before the addition of 3 ml of accutase (Chemicon) to detach cells from the flask. The flask was gently tapped to detach the remaining cells stuck to the flask. Accutase cell suspension was aspirated and transferred to a 15 ml falcon tube and centrifuged at 500 rpm for 5 minutes. Supernatant was discarded, and the cell pellet washed with 1 ml of growth medium. The cell pellet was then suspended in 1 ml of growth medium. The cell suspension (200  $\mu$ l) was then added to a T25 culture flask containing 5 ml of growth medium.



#### **4.6 Transfection of BMSSCs, P19 and NIH3T3**

One day prior to transfection, cells were seeded at  $4-8 \times 10^5$  cells per well into T5 6 well tissue culture plates (Gibco) containing 2 ml of growth medium per well. Cells at 90-95% confluency were transfected with plasmid DNA (4  $\mu$ g) isolated as described in section 1.1, and mixed with 250  $\mu$ l of Opti-MEM I reduced serum medium (Invitrogen). A 10  $\mu$ l volume of lipofectamine (Invitrogen) was diluted in 250  $\mu$ l of Opti-MEM I reduced serum medium and incubated for 5 minutes at room temperature. Diluted DNA was combined with diluted lipofectamine (total volume 500  $\mu$ l), mixed gently and incubated for 20 minutes at room temperature. Plasmid DNA – lipofectamine complex (500  $\mu$ l) was added to each well containing cells plus medium. The contents were mixed by gently rocking the plate back and forth. Cells were incubated at 37°C in a 5% carbon dioxide incubator for 18-48 hours prior to testing for gene expression. After 24 hours, transfection medium was replaced with growth medium containing 1% gentamycin for stable transfection selection.

#### **4.7 Selecting Pax7+ stable clones**

Cells were visualised under an inverted microscope and individual clones containing *Pax7d*, or vector alone were selected from each cell line using sterile swabs. Individual clones were propagated in growth medium containing 1% gentamycin. Prior to placing the cells in differentiation medium, expression of *Pax7* or vector was confirmed in each clone by RT-PCR and immunocytochemistry. For each cell line, clones expressing high levels of *Pax7* or vector alone were selected for further experimentation. For each cell line, a total of two clones expressing *Pax7* and two clones containing vector alone were chosen for further experiments, representing a

total of 4 clones (two Pax7+ and two vector alone) per cell line. Cell transfected vector alone did not survive the selection process so untransfected cells were utilised as controls.

#### **4.8 Differentiating the cells**

Differentiation medium consisted of 42%  $\alpha$ -MEM, 42% F12, 15% knockout serum and 100U/ml penicillin/streptomycin/fungizone. Before transferring cells to differentiation medium, the cells were analysed using RT-PCR and immunocytochemistry and termed Day 0. Cells were then transferred and cultured in differentiation medium for 7 days, after which time they were collected for RNA extraction and RT-PCR analysis or fixed for immunocytochemistry and termed Day 7.

#### **4.9 RNA extraction for RT-PCR**

RNA from the cells was extracted using PureLink Micro-to-midi Total RNA purification system (Invitrogen) according to the manufactures instructions. Briefly, cells were pelleted by centrifugation of accutased cells at 500 rpm for 5 minutes. The cell pellet was washed with PBS and centrifuged at 500 rpm for 5 minutes. The cells were lysed with 200  $\mu$ l of lysis buffer containing 1%  $\beta$ -mercaptoethanol and mixed gently. An equal volume of 70% RNase free ethanol was added and mixed gently. Then 400  $\mu$ l of lysate was transferred to an RNA binding spin cartridge and centrifuged at 12000 rpm for 15 seconds. The flow through was discarded. A 350  $\mu$ l volume of wash buffer I was added to the spin cartridge centrifuged at 12000 rpm for 15 seconds and flow through discarded. A further 700  $\mu$ l of wash buffer I was added to the spin cartridge, centrifuged at 12000 rpm for 15 seconds and the flow through discarded. The spin cartridge was placed in a new RNase free wash tube. 500  $\mu$ l of

wash buffer II was added, and then the cartridge was centrifuged at 12000 rpm for 15 seconds and the flow through discarded. A further 500 µl of wash buffer II was added, the cartridge was again centrifuged at 12000 rpm for 15 seconds and flow through discarded. The spin cartridge was centrifuged at 12000 rpm for a further 1 minute to dry the RNA binding membrane. The spin cartridge was placed in an RNase free collection tube. RNase free water (50 µl) was placed on the RNA column for 1 minute, and then RNA was eluted by centrifuging at 12000 rpm for 2 minutes. RNA was assessed by gel electrophoresis for quality and the concentration was determined by spectrophotometry. RNA was stored at -80°C until used in RT-PCR experiments.

RT-PCR was used to assess Pax7 expression and neural differentiation in the cell line prior to and after transfection. A total of 50 ng of RNA was converted to cDNA using an Omniscript Reverse Transcriptase Kit (Qiagen): a 20 µl reaction contained 4 units Omniscript reverse transcriptase, 1x RT buffer (15 mM MgCl<sub>2</sub>), dNTPs (5 mM of each dNTP) RNase inhibitor (10 units/µl) and random hexamers as primers (10 µM). The reaction was incubated at 37°C for 1 hour, and 2 µl of cDNA was utilised in each downstream PCR. PCR was performed in a 20 µl reaction which contained 1x PCR buffer (15 mM MgCl<sub>2</sub>), 1x Q-solution, 10 µM forward primer, 10 µM reverse primer, 10 µM dNTPs, 0.5 units of Taq polymerase per reaction and 2 µl cDNA. The PCR conditions for all reactions were: 94°C for three minutes followed by 45 cycles comprising 94°C for 30 seconds, 52°C- 57°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 10 minutes. Annealing temperatures ranged from 52°C - 57°C and were specific for each set of primers used in the PCR reactions.

The PCR products were visualised on 1% agarose gels stained with ethidium bromide and analysed using the GelDoc system. All RT-PCR reaction products were

semi-quantified relative to GAPDH expression levels using software Quantity One (BioRad). Gene expression levels was analysed in duplicates for each clone of each stem cell type.

For each clone transfected with *Pax7*, or for the cells transfected with vector alone, PCRs were performed on the isolated RNA with gene specific primers (Table 4.1). The genes tested were *Pax7*, neural markers (*Neurofilament*, *Beta III tubulin*, *MAP2*, *nestin*, *Engrailed 2*, *NCAM* and *Ephrin A2*) or the glial marker *GFAP*, and stem cell markers (*CD44*, *CD90* and *CD11B*). The summary of gene specific primers used in RT-PCR reaction is shown in Table 4.1.

Table 4.1. A summary of the genes and primers used in RT-PCR reactions to test neural differentiation of transfected cells.

Marker	Forward primer 5' to 3'	Reverse primer 5' to 3'	Product size
Pax7	CACCGACTCTGGATTCGTCTCCAGCGTG	ACAACCATGAGAAACACGGAGC	279 bp
GFAP	TCCTGGAACAGCAAAACAAG	CAGCCTCAGGTTGGTTTCATC	224bp
Engrailed 2	TGGATGGAGTGCTCAAAGC	ACCTGTTGGTCTGAAACTCAGC	273 bp
Nestin	GAGTCAGATCGCTCAGATCC	AAGAGAAGGATGTTGGGCTGAG	248 bp
Beta III tubulin	TGGAGCGCATCAGCGTATACTAC	TCTCACACTCTTCCGCACG	246bp
NCAM	TACCCTCACCATCTACAACG	TGTTGGACAGGACTATGAACCG	278 bp
Neurofilament	CAGCAGTTGGAAAATGAACTTC	GATGGCTGTGAGGGTTTCTTCC	342 bp
CD44	TGACAGGTTCCATTCACCCTCG	AGGTACTGTTGAAAGCCTGGC	276 bp
CD11B	ATGGGCTCAGGAAACAAC TG	GACCACATTCTGTCCAAAGC	259 bp
Ephrin A2	CAACGCTGACCGATACGC	CATAAACCTTGAGTCGCTGGC	413 bp
GAPDH	GTGAAGGTCGGTGTGAACG	ATTTGATGTTAGTGGGGTCTCG	233 bp
CD90	AATCCAAGTCGGAAC TCTTGGC	ATCCTTGGTGGTGAAGTTGGC	322 bp

#### 4.10 Fixing cells for Immunocytochemistry

The cells grown for 0 days or 7 days in differentiation medium were cultured in 8 well immunocytochemistry chamber slides (Nunc). After 24 hours the cells were washed with TBS three times and fixed in 4% paraformaldehyde for 10 minutes. The cells were washed three times with Tris buffered saline (TBS) and quenched in 3% hydrogen peroxide for 10 minutes. The cells were again washed with TBS three times. Non-specific binding was blocked by incubating the cells in TBS / 0.1%

Triton-X 100 / 3% normal goat serum (NGS) (Invitrogen) and 3% fetal calf serum (FCS) (Invitrogen) for 1 hour at room temperature. Primary antibodies were applied overnight at 4°C; primary antibodies were diluted in TBS / 0.1%TX / 1% NGS / 1% FCS. Table 4.2 gives a summary of the primary antibodies and the dilutions used for immunocytochemical analysis of the cells.

Table 4.2. A summary of the primary antibodies used and their cell type specificity.

Antibody	Company	Raised in/clonality	Dilution	Cell type detected
Pax7	DSHB	Mouse/monoclonal	1/10	Transfected cells
NeuN	Abcam	Mouse/monoclonal	1/250	Neural
GFAP	Chemicon	Rabbit/polyclonal	1/500	Astrocyte
Nestin	DSHB	Mouse/monoclonal	1/100	Stem cell
Beta III Tubulin	Abcam	Mouse/monoclonal	1/500	Neural
Ki-67	Abcam	Rabbit/polyclonal	1/250	Proliferation
Neurofilament	Chemicon	Rabbit/polyclonal	1/500	Neural
OX42	Abcam	Mouse/monoclonal	1/250	Dendritic macrophage

Primary antibody was removed and the cells washed with TBS. Secondary antibody, anti mouse/rabbit (anti-IgG) linked to biotin (LSAB kit DAKO) was applied for one hour at room temperature, followed by the tertiary antibody, streptavidin linked to horse-radish peroxidise (HRP) (LSAB Kit DAKO), for one hour at room temperature. The tertiary antibody was removed; the cells were washed with TBS three times followed by TNS twice. Cells were visualised by a colour reaction using diaminobenzidine (DAB) (DAKO). The cells were finally washed with TNS, and cover-slipped using aqueous mounting medium to avoid losing stained cells.

Primary antibodies used were: Pax7 (DSHB); neural markers Neurofilament (Chemicon), NeuN (Abcam) and Beta III tubulin (Abcam); the cell proliferative marker used was Ki67 (Chemicon); astrocyte marker was Glial fibrillary acidic

protein GFAP (Chemicon); the neural precursor marker Nestin (DSHB) and the macrophage / dendritic cell marker OX42 (Abcam), (see Table 4.2). The cells were visualised with an Olympus microscope and photographed using a Leica DC300 camera.

## **CHAPTER 5**

### **RESULTS**

5.1     Cloning Pax7 into the expression vector

*Pax7d* previously cloned into the *pcDNA3.1 D/V5 HIS-TOPO* vector or vector alone were amplified in *E.Coli*. Purified vector DNA was run on a 1% agarose gel to check the DNA purity. The total size of the vector was 5514 bps and the *Pax7* insert was 1560 bps, thus the expected size of the vector plus the insert was 7074 bps. Circular plasmids adopt multiple structural conformations, which in turn affect the rate at which they migrate in an agarose gel during electrophoresis. Thus three bands were visible on the gel, implying that three different structures were adopted by the circular *pcDNA3.1* vector.



Figure 5.1. Purified vector DNA containing *Pax7* or vector alone control, were electrophoresed on 1% agarose gel adjacent to a 1 Kb ladder. The size of the purified product was approximately 7 kilo bases. Three bands are clearly visible, as expected for circular plasmids, which adopt different structural conformations in solution.



5.2     PCR to confirm Pax7 insertion into the vector

PCR was performed on the purified plasmid DNA to confirm the *Pax7* insertion. Primers specific to the vector and insert, F4 and BGH, were used for the PCR. The expected PCR product size was 1748 bp. For the forward primer, only four base pairs are complementary to the vector and the rest of the primer spans the start of *Pax7*. The reverse primer was complementary to the vector; at a position a further 184 base pairs from the end of the *Pax7* insert. Thus the PCR product was 4bp plus 1560bp plus 184bp which equals 1748bp.

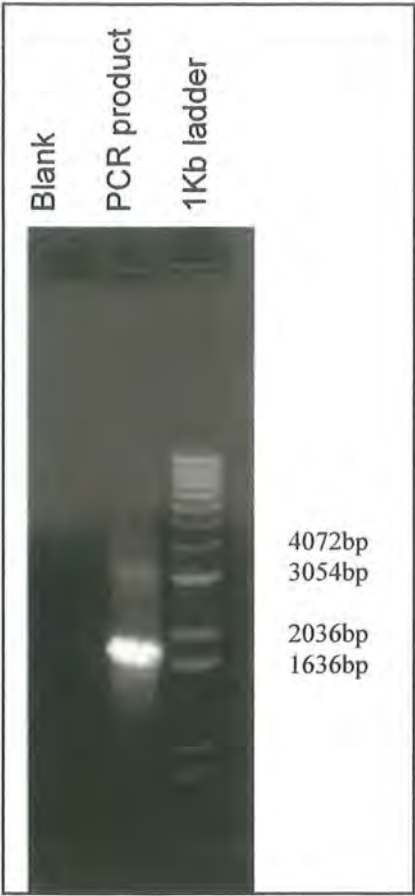


Figure 5.2. PCR was performed on purified vector DNA containing *Pax7*. The product size as expected was 1748 bp. There was no DNA detected in the blank (no DNA control).

### 5.3 Confirmation of Pax7 expression in transfected and selected cells

#### P19 cells

After cells were transfected and stably selected, the *Pax7* insert was confirmed by immunocytochemistry. Untransfected P19 cells did not express any endogenous *Pax7*. As expected transfected cells express *Pax7*, confirming *Pax7* expression from the vector. P19 cells transfected with vector alone did not survive gentamycin selection; all the P19- vector cells died due to unsuccessful transfection. Therefore untransfected P19 cells were used as a comparative control.

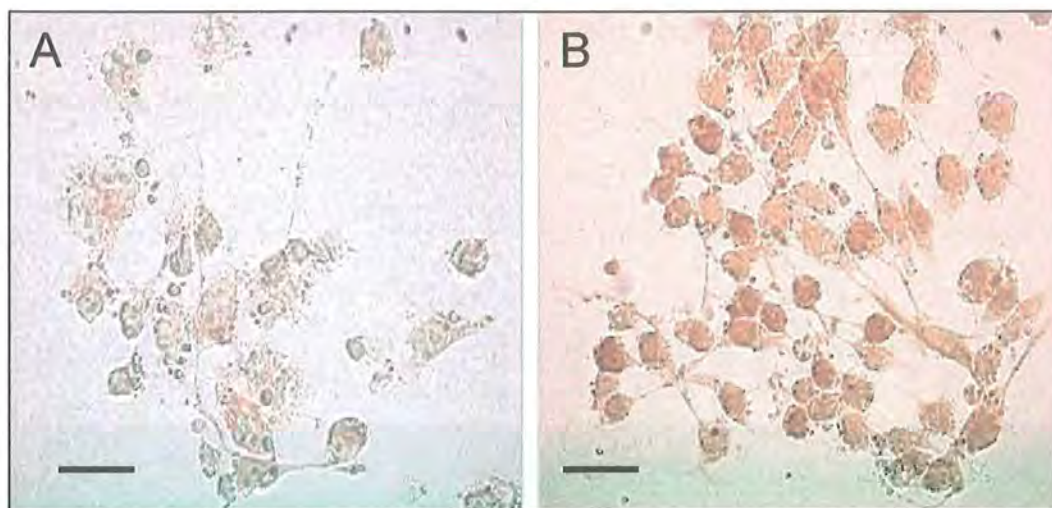


Figure 5.3. A: untransfected P19 cells showing no expression of *Pax7*. B: P19 cells transfected with *Pax7* detected by immunocytochemistry with Pax7 antibody. Every single transfected cell expressed *Pax7*. A representative sample of *Pax7* transfected cells from clone one is shown here. Similar results were obtained for clone two. Scale bar 10µm.

The success of *Pax7* transfection was crucial for the remaining experiments; without expression of *Pax7* in the cells, the down stream target genes regulated by *Pax7* would not be activated or would only be regulated at basal level. Thus we would not be able to assess Pax7 induced stem cell differentiation into specific neurons.

5.4 Gene expression in P19 cells

After RNA was extracted and converted to cDNA, PCR was performed to determine which genes were expressed by untransfected P19 cells, termed basal gene expression. Stem cells are known to express low levels of a wide variety of genes. Thus they have wide potential for differentiation, and differentiation is often driven by micro-environmental factors surrounding the cells (Blondheim et al., 2006). When a cell starts to differentiate along a particular cell lineage, genes related to that cell type are up-regulated and the rest of the basal level gene expression is shut down. Here, testing for basal level gene expression patterns in untransfected P19 cells indicated that a wide variety of genes were expressed in P19 cells at various expression levels (Refer to figure below).

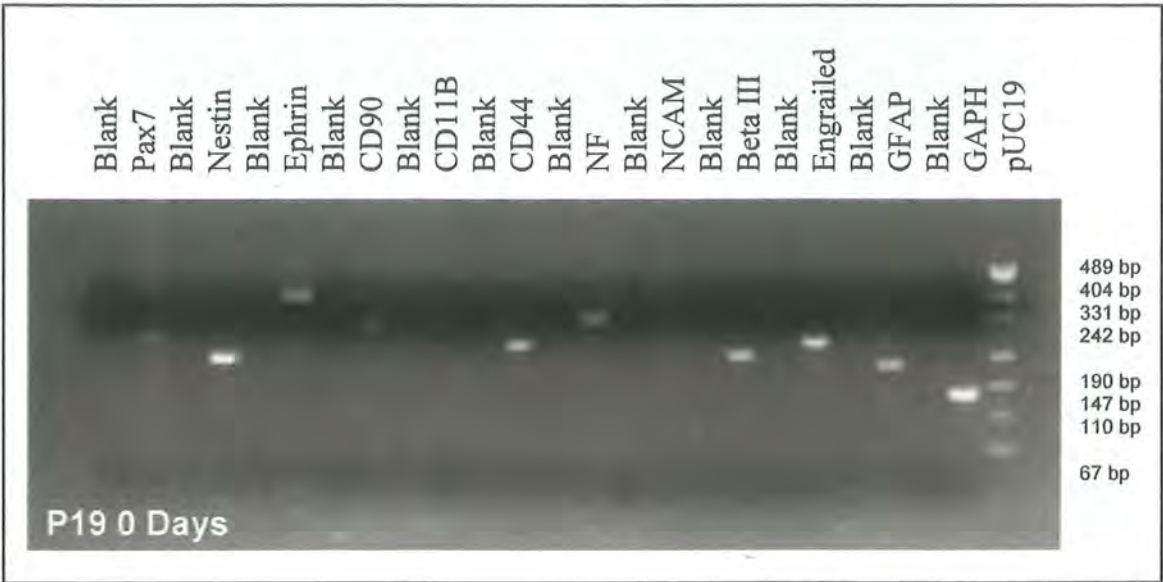


Figure 5.4. RT-PCR for P19 cells to detect basal level gene expression in untransfected cells. Variable band intensity on the gel for several genes indicated varied expression levels relative to *GAPDH*. Untransfected P19 cells did not express *CD11B* and expressed only very low levels of *Pax7* and *NCAM*. Blank, no DNA control, for each set of primers was negative.



5.5 Gene expression in P19 + Pax7 transfected cells

RT-PCR performed on RNA from P19 cells transfected with *Pax7*, indicated definitive suppression of *GFAP* and *Nestin* expression. When the intensity of bands was quantified, there was a significant difference in gene expression patterns compared to basal level gene expression levels present in P19 cells (Table 5.1).

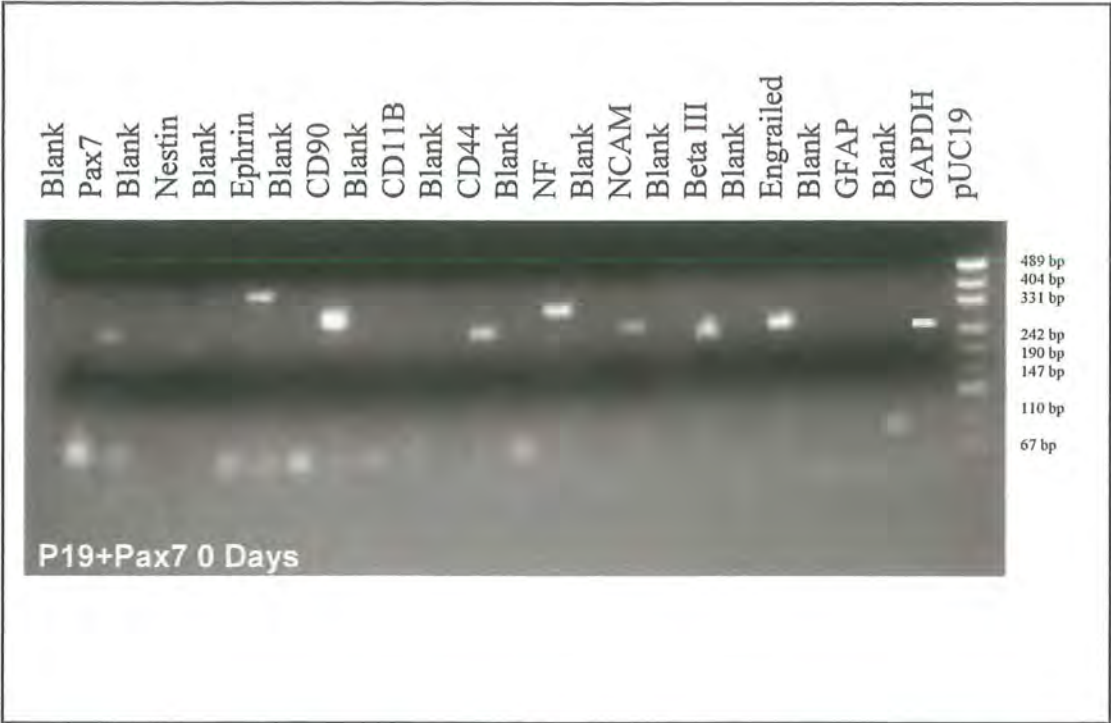


Figure 5.5. The gene expression patterns in P19 cells transfected with *Pax7* differed from those in untransfected P19 cells. Expression of *GFAP* and *nestin* expression were significantly reduced in *Pax7*+ cells. The increased expression of *Neurofilament* and *Beta III tubulin* in *Pax7*+ cells indicates that P19+*Pax7* cells have commenced differentiation along a neurogenic rather than an astrocytic lineage. *Pax7* expression levels increased as did levels of *Ephrin A2* and *Engrailed 2* – all markers of dorsal midbrain neurons.

5.6 Gene expression in P19 + Pax7 transfected cells after 7 days in differentiation medium

P19 and P19+Pax7 cells were grown in differentiation medium containing  $\alpha$ -MEM, F12, knockout serum and antibiotic without EGF and FGF (see section 4.8). The growth factors were withdrawn to ensure that the changes in gene expression observed after *Pax7* transfection were not due to growth factors. Moreover the growth factors were used for rapid proliferation of cells, therefore to differentiate the transfected cells, growth factors were removed from the culture medium.

The gene expression of P19 cells transfected with *Pax7* did not change after seven days in medium without growth factors EGF and FGF. Only *Pax7* expression was dramatically increased. Most other genes remained up or down regulated in patterns similar to that seen in transfected cells at day 0, but gene expression for most of the genes was slightly decreased when analysed by band intensity relative to GAPDH.

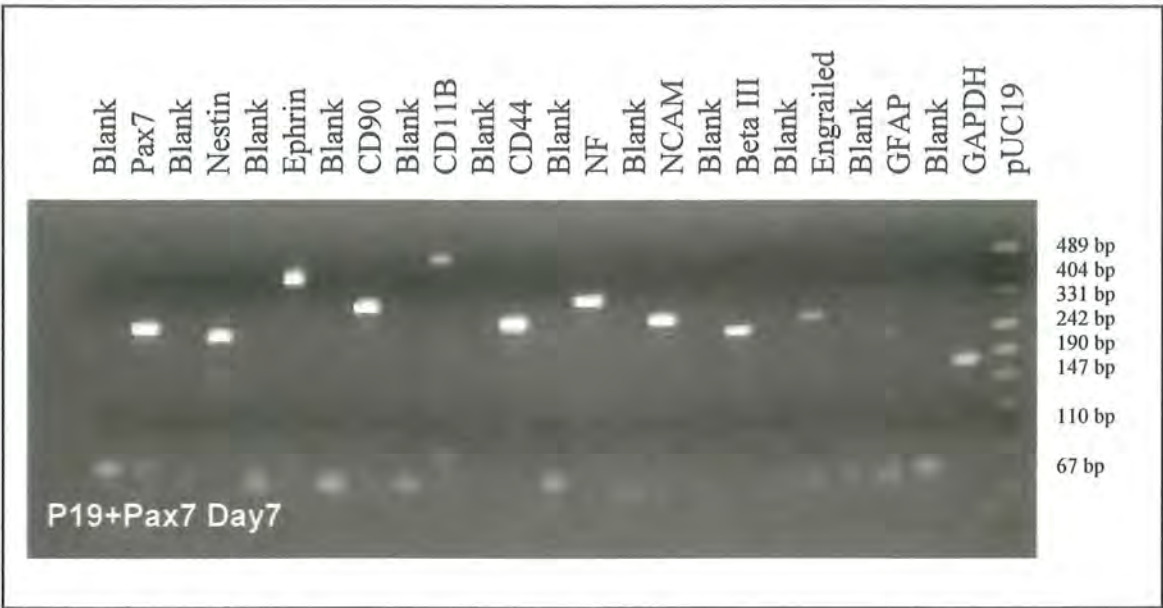


Figure 5.6. RT-PCR for P19 cells transfected with *Pax7* and grown in differentiation medium for 7 days. There was no great difference in gene expression relative to *Pax7* transfected cells at day 0. Most genes were in fact slightly down regulated when band intensity was analysed relative to GAPDH.

Table 5.1. Semi-quantitative analysis of gene expression levels in P19 and P19+*Pax7* transfected cells. Quantity One (Bio-Rad) was used to analyse band intensity relative to the GAPDH PCR product and used as a measure of fold difference in gene expression levels.

	P19 cells	P19 Pax7 ( 0 days)	P19 Pax7 ( 7 Days)	Fold Difference
Pax7	0.15	0.66	1.58	10
Nestin	1.00	1.25	1.56	1.5
Ephrin A2	0.37	2.54	1.44	6
CD90	0.11	2.94	1.57	27
CD11B	Nil	Nil	0.60	0.5
CD44	0.65	1.82	1.50	3
Neurofilament	0.30	2.79	1.55	9
NCAM	Nil	1.03	1.47	1.5
Beta III tubulin	0.68	1.94	1.49	2.5
Engrailed 2	0.86	2.85	0.54	3
GFAP	0.60	0.52	0.16	0.25
GAPDH	1.00	1.00	1.00	1

In cells transfected with *Pax7*, *Pax7* expression increased at day 0 and was further increased after incubation in differentiation medium for 7 days. Neural genes were also up regulated, including nestin which is a neural progenitor marker. *GFAP* levels decreased after *Pax7* transfection, whereas the mature neural markers *Ephrin A2*, *Neurofilament*, *NCAM*, *Beta III tubulin* and *Engrailed* increased in transfected cells relative to untransfected cells. The results indicate that the P19 stem cells are likely to differentiate into neurons after transfection with *Pax7*.



5.7 Immunocytochemistry of P19 cells and P19+Pax7 cells

1. GFAP

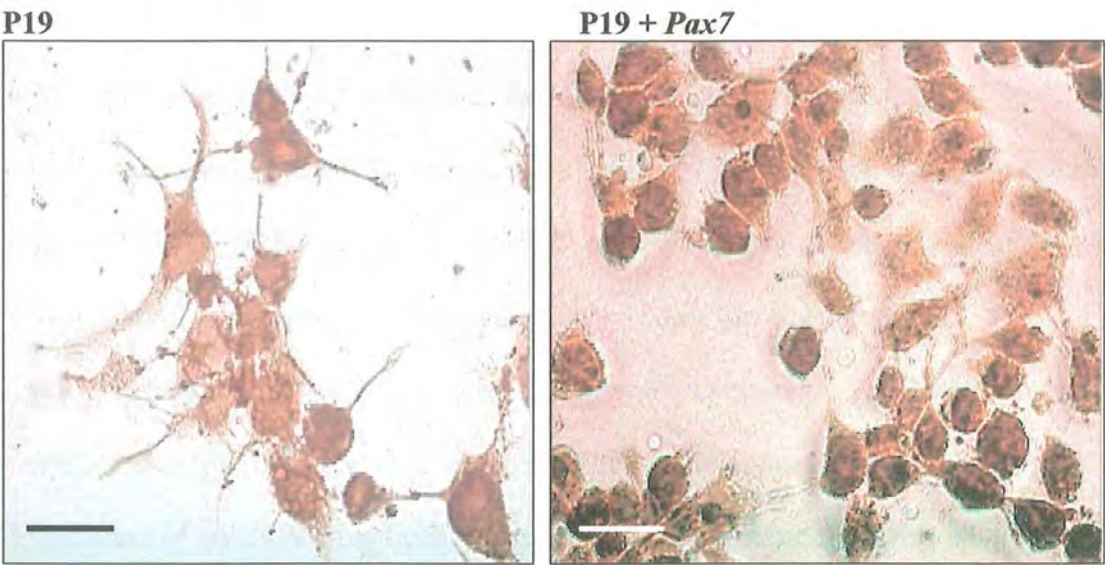


Figure 5.7. Expression of GFAP in untransfected P19 cells and *Pax7* transfected P19 cells. Scale bar 10µm.

2. NeuN

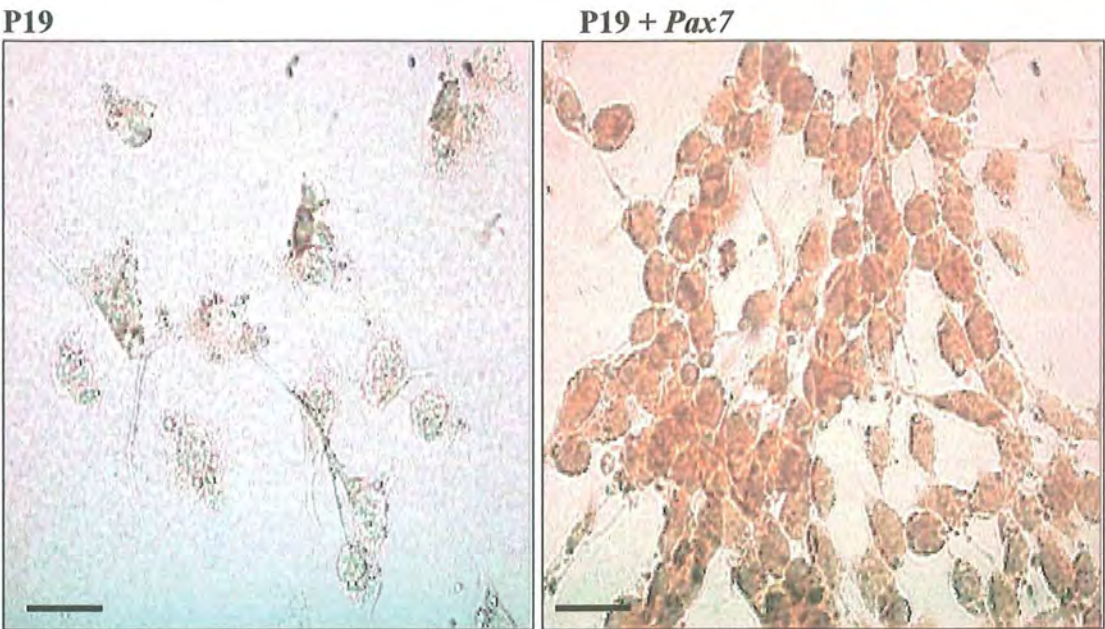


Figure 5.8. Expression of the neuronal marker NeuN in untransfected P19 cells and *Pax7* transfected P19 cells. Scale bar 10µm.

Immunocytochemistry was performed to detect protein levels in the cells transfected with *Pax7* relative to untransfected control cells. Pax7 protein was clearly evident in transfected cells while no Pax7 was detected in untransfected cells (Figure 5.3). Furthermore both transfected and untransfected cells expressed GFAP, an astrocyte marker (Figure 5.7), however the levels of GFAP in each cell may differ as indicated by RT-PCR. The levels of GFAP protein in *Pax7* transfected cells appears lower relative to untransfected P19 cells, however this was not quantified.

Expression of NeuN, a marker for mature neurons, is evident in *Pax7* transfected cells but was not present in the untransfected cells (Figure 5.8). Expression of NeuN is an indication that the cells have exited from the cell cycle and maturation and differentiation towards a neuron has commenced (Sarnat et al., 1998). All the transfected cells expressed NeuN and therefore were on a path of neuronal differentiation as suggested by protein expression.



### 3. Nestin

P19



P19 + *Pax7*

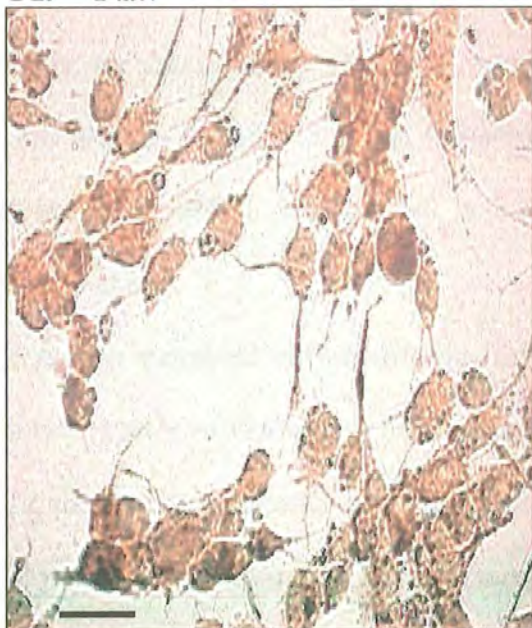
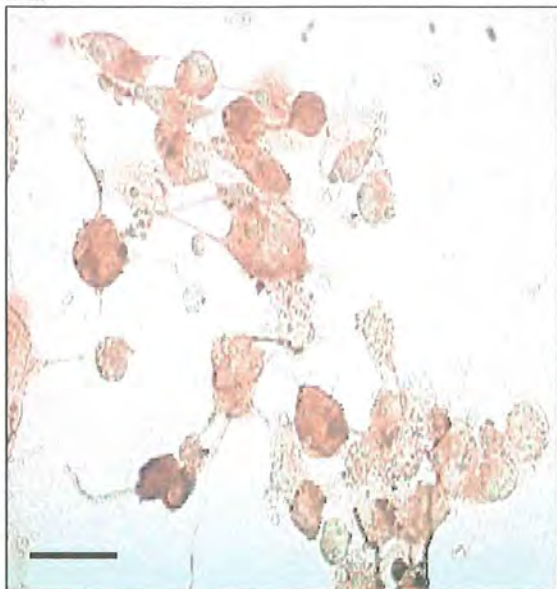


Figure 5.9. Expression of neural precursor marker Nestin in untransfected P19 cells and *Pax7* transfected P19 cells. Scale bar 10 $\mu$ m.

### 4. Beta III Tubulin

P19



P19 + *Pax7*



Figure 5.10. Expression of Beta III tubulin in untransfected P19 cells and *Pax7* transfected P19 cells. Scale bar 10 $\mu$ m.

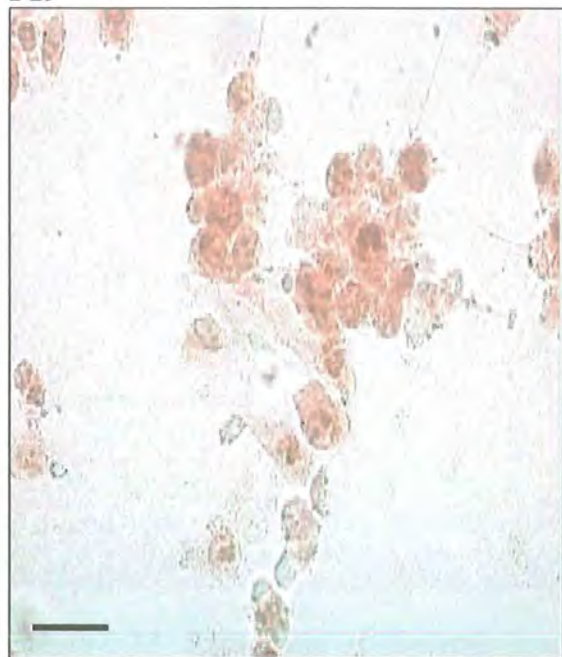
Nestin expression appeared reduced in transfected cells compared to untransfected control cells. P19 stem cells showed expression of nestin at a basal level, but as the cell differentiated towards a neural lineage, nestin expression appeared diminished (Figure 5.9). Nestin is an intermediate filament protein expressed predominantly by neural stem cells and its thought to be an important marker of early neural cells (Wislet-Gendebien et al., 2003). When a cell is fully differentiated and matured, nestin is not expressed at all.

Beta III tubulin is a cellular structural protein expressed as five isoforms. Beta III tubulin is specifically expressed as a structural protein exclusively in neurons (Lee et al., 1990). Levels of Beta III tubulin in the *Pax7* transfected cells increased, indicating that the transfected cells were differentiating towards a neural lineage (Figure 5.10). Not all untransfected cells expressed Beta III tubulin whereas, by contrast, all transfected cells expressed high levels of Beta III tubulin.



## 5. Ki67

P19



P19 + *Pax7*

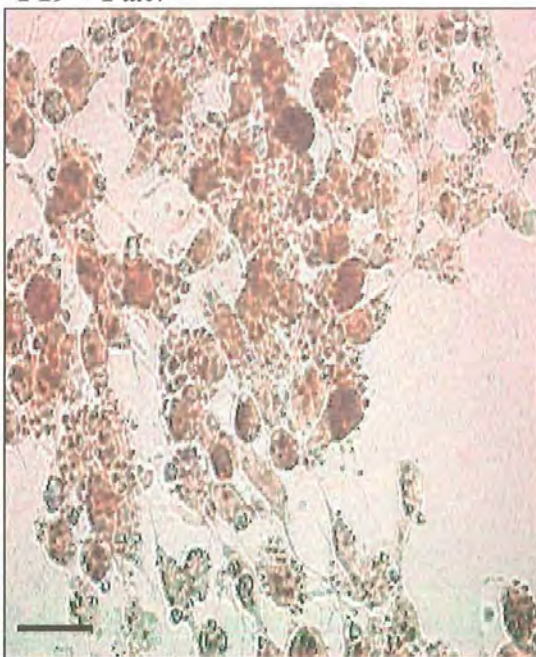


Figure 5.11. Expression of proliferative marker Ki67 in untransfected P19 cells and *Pax7* transfected P19 cells. Scale bar 10 $\mu$ m.

## 6. Neurofilament

P19



P19 + *Pax7*

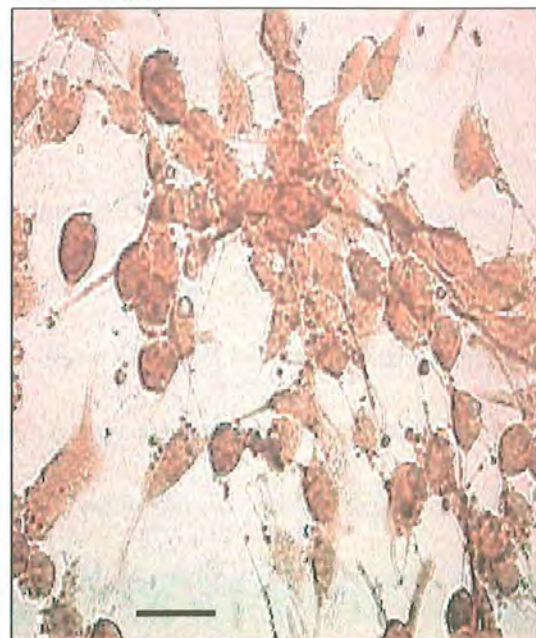


Figure 5.12. Expression of neuronal marker Neurofilament in untransfected P19 cells and *Pax7* transfected P19 cells. Scale bar 10 $\mu$ m.

## 7. OX42

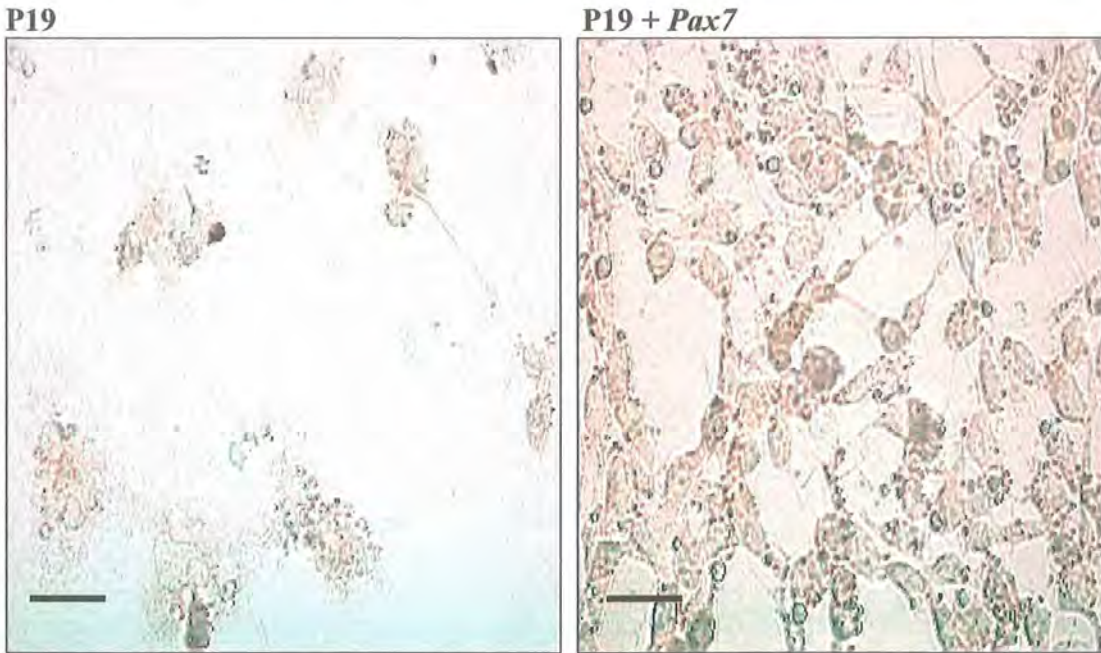


Figure 5.13. Expression of dendritic marker OX-42 in untransfected P19 cells and *Pax7* transfected P19 cells. Scale bar 10 $\mu$ m.

Ki67 is a protein that is expressed in the nuclei of proliferating cells. Absent only in resting cells, cells in the G1, S, G2, and M phase of the cell cycle express this marker (Keng and Siemann, 1998). Both *Pax7* transfected and untransfected cells expressed nuclear Ki67 (Figure 5.11), indicating that the cells remained proliferative after *Pax7* transfection.

Neurofilament is a structural protein present in neurons, and is expressed by both early neuroblasts as well as post mitotic neurons. It is indicative of cell differentiation towards a neural lineage. Neurofilament is a filament protein consisting of three forms: light, medium and heavy neurofilament, all of which are structural proteins found in the cytoplasm of neurons. In *Pax7* transfected cells neurofilament was observed in the cell body and projecting cell processes (Figure 5.12). In untransfected cells, the expression of neurofilament was restricted to the region around the nucleus, denoting undifferentiated cells.

OX-42 is a marker of dendritic macrophages. Both transfected and untransfected cells did not express any OX-42 (Figure 5.13).



5.8 Immunocytochemistry of NIH3T3 cells and Pax7+ NIH3T3 cells

Immunocytochemistry was also performed on NIH3T3 cells and on NIH3T3 cells transfected with *Pax7* to determine expression of *Pax7* and selected genes. The cells were transfected with *Pax7d*, and stable clones were selected and analysed for *Pax7* expression as described previously in section 4.6. Vector transfection was performed but NIH3T3 cells with vector alone did not survive gentamycin selection. Untransfected NIH3T3 cells were used as comparative controls.

1 Pax7

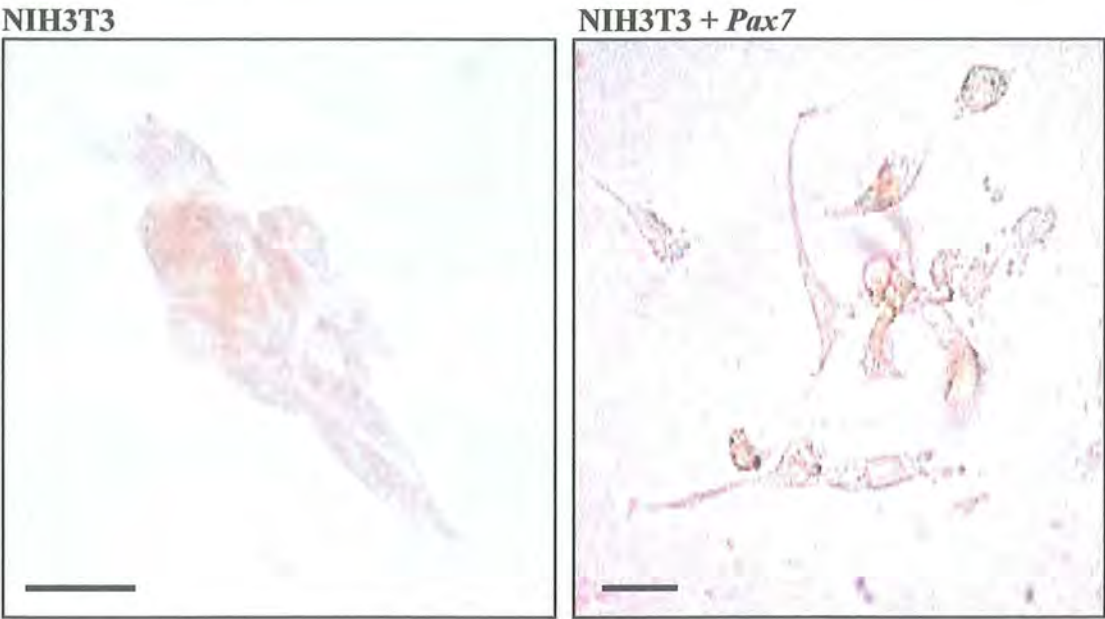


Figure 5.14. Expression of *Pax7* in NIH3T3 untransfected cells compared to *Pax7* transfected NIH3T3 cells. Scale bar 10µm.

## 2 GFAP

NIH3T3



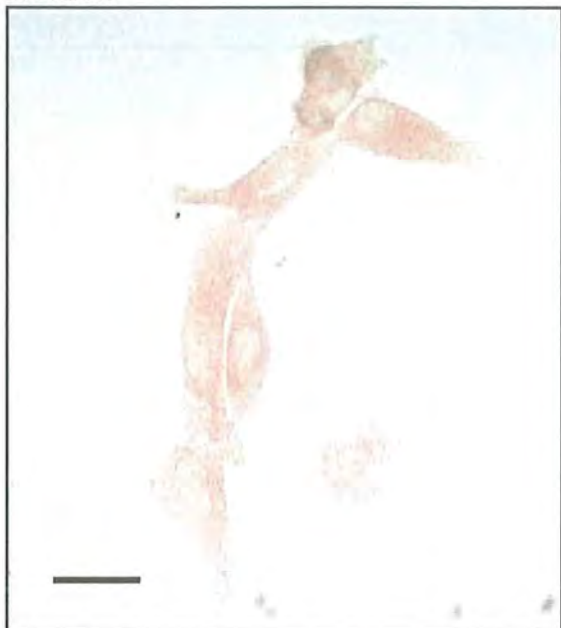
NIH3T3 + *Pax7*



Figure 5.15. Expression of GFAP in NIH3T3 untransfected cells compared to *Pax7* transfected NIH3T3 cells. Scale bar 10 $\mu$ m.

## 3 NeuN

NIH3T3



NIH3T3 + *Pax7*



Figure 5.16. Expression of NeuN in NIH3T3 untransfected cells compared to *Pax7* transfected NIH3T3 cells. Scale bar 10 $\mu$ m.

#### 4 Nestin

NIH3T3



NIH3T3 + *Pax7*

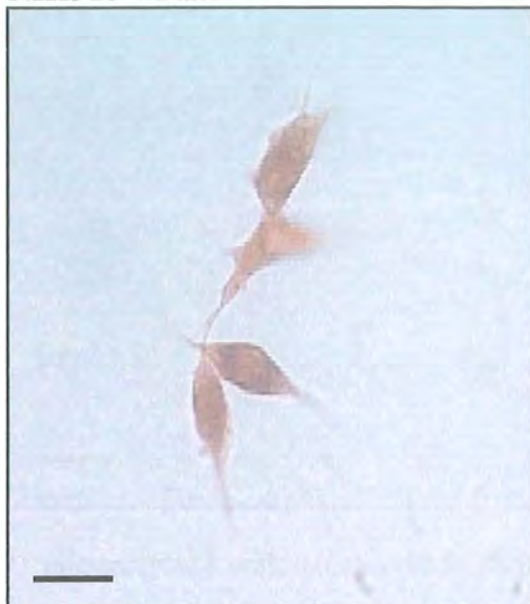
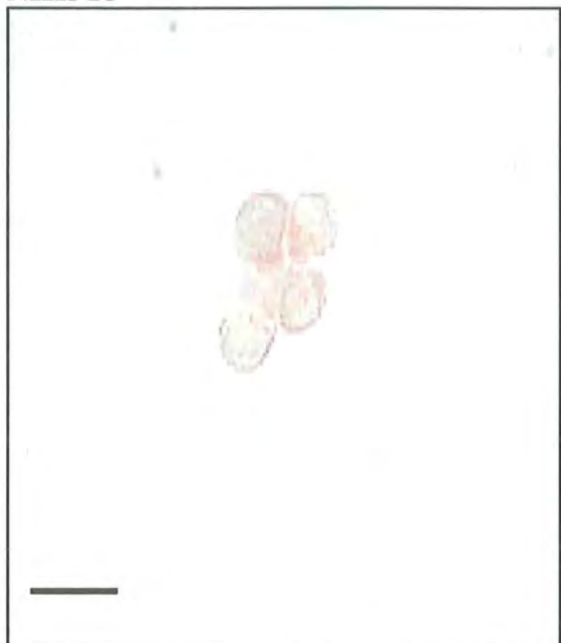


Figure 5.17. Expression of Nestin in NIH3T3 untransfected cells compared to *Pax7* transfected NIH3T3 cells. Scale bar 10 $\mu$ m.

#### 5 Beta III tubulin

NIH3T3



NIH3T3 + *Pax7*

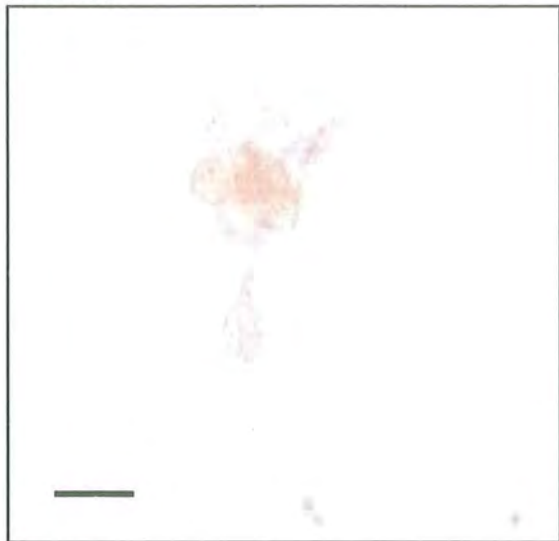


Figure 5.18. Expression of beta III tubulin in NIH3T3 untransfected cells compared to *Pax7* transfected NIH3T3 cells. Scale bar 10 $\mu$ m.



6 Ki67

NIH3T3



NIH3T3 + *Pax7*



Figure 5.19. Expression of Ki67 in NIH3T3 untransfected cells compared to *Pax7* transfected NIH3T3 cells. Scale bar 10 $\mu$ m.

7 Neurofilament

NIH3T3



NIH3T3 + *Pax7*

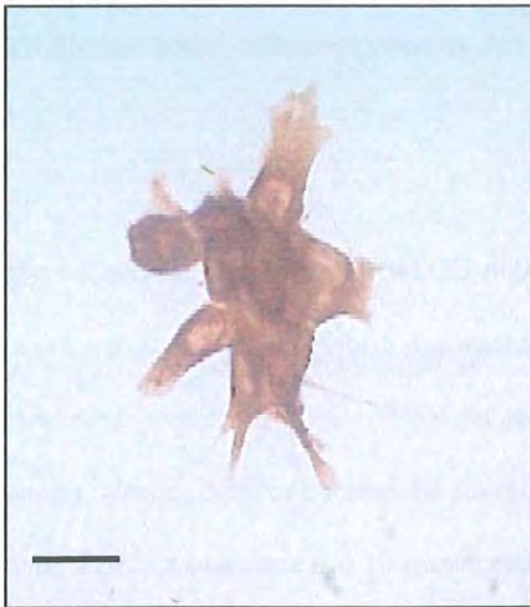


Figure 5.20. Expression of Neurofilament in NIH3T3 untransfected cells compared to *Pax7* transfected NIH3T3 cells. Scale bar 10 $\mu$ m.

## 8 *OX-42*



Figure 5.21. Expression of OX-42 in NIH3T3 untransfected cells compared to *Pax7* transfected NIH3T3 cells. Scale bar 10μm.

There was no difference between gene expression patterns of NIH3T3 cells and P19 cells. Both untransfected cell lines were negative for *Pax7* while transfected cell lines were positive for *Pax7*; *Pax7* protein was present in 80% - 100% of the cells. Expression of neural markers neurofilament, Nestin, NeuN and Beta III tubulin increased in NIH3T3 cells after transfection with *Pax7* as observed in P19 transfected cells. In NIH3T3 transfected cells Ki67 expression appeared increased relative to untransfected cells. OX-42 was negative in both transfected and untransfected NIH3T3 cells. Since immunocytochemistry results were consistent with those obtained from P19 cells transfected with *Pax7*, RT-PCR was not performed on NIH3T3 cells.

Similar results in P19 and NIH3T3 cells confirmed that previous experiments (Ziman et al., 2001) in which it was observed that *Pax7* induced differentiation of P19 cells along a neurogenic lineage, were not due to artefacts or an innate property of the cell line.

5.9 Immunocytochemistry for rBMSSCs

Immunocytochemistry was performed on rBMSSCs to assess *Pax7* and neural gene expression in untransfected cells. The transfected cells survived two weeks after transfection but died during selection, therefore no RT-PCR or immunocytochemistry could be performed on the *Pax7* transfected cells.

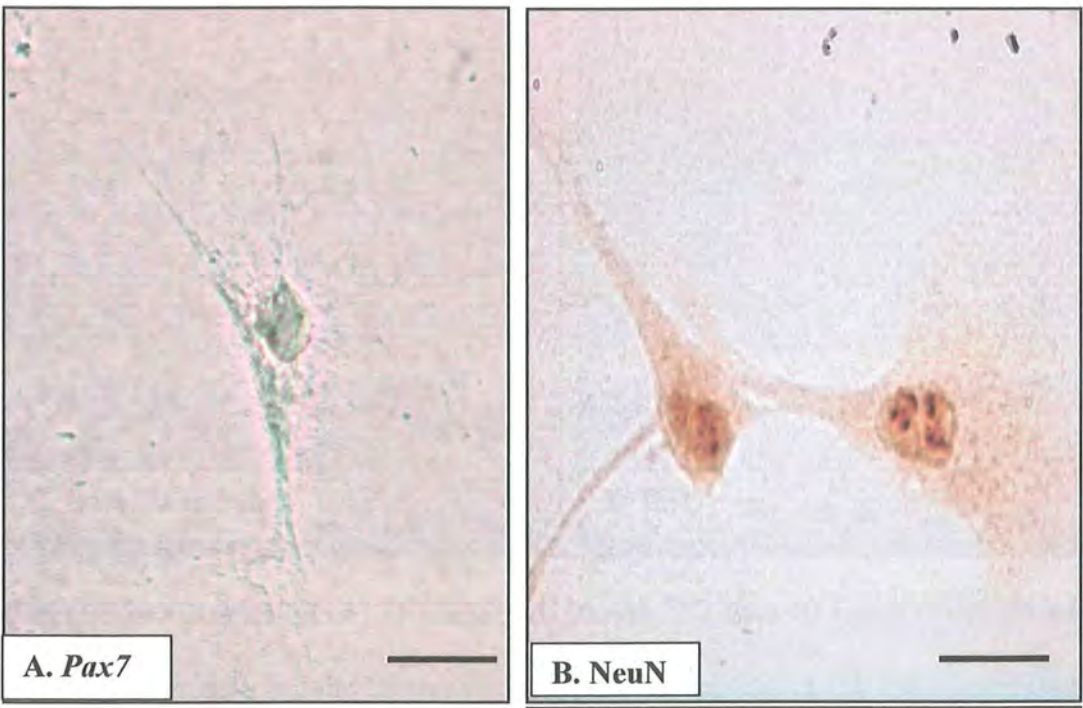


Figure 5.22. Expression of (A) *Pax7* and (B) NeuN in untransfected BMSSCs. No *Pax7* expression was evident whereas NeuN expression was clearly present in the nucleus. Scale bar 10µm.



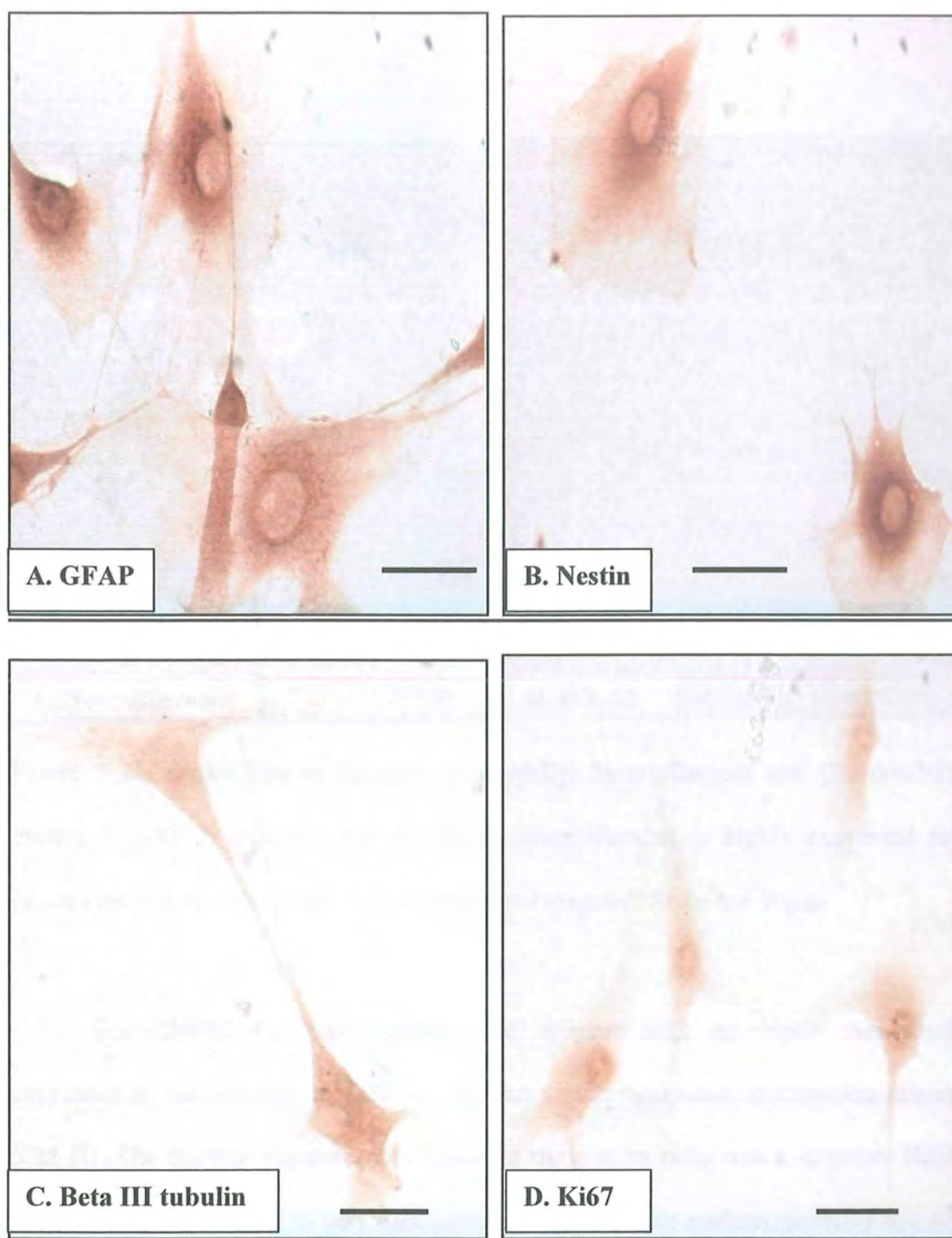


Figure 5.23. Expression of (A) GFAP, (B) Nestin, (C) Beta III tubulin and (D) Ki67. GFAP was present around the nucleus, with little staining in the cytoplasm. Nestin was present in the cytoplasm and around the nucleus. Beta III tubulin was present throughout the cell. Proliferative marker Ki67 was specifically restricted to the nucleus. Scale bar 10 $\mu$ m.

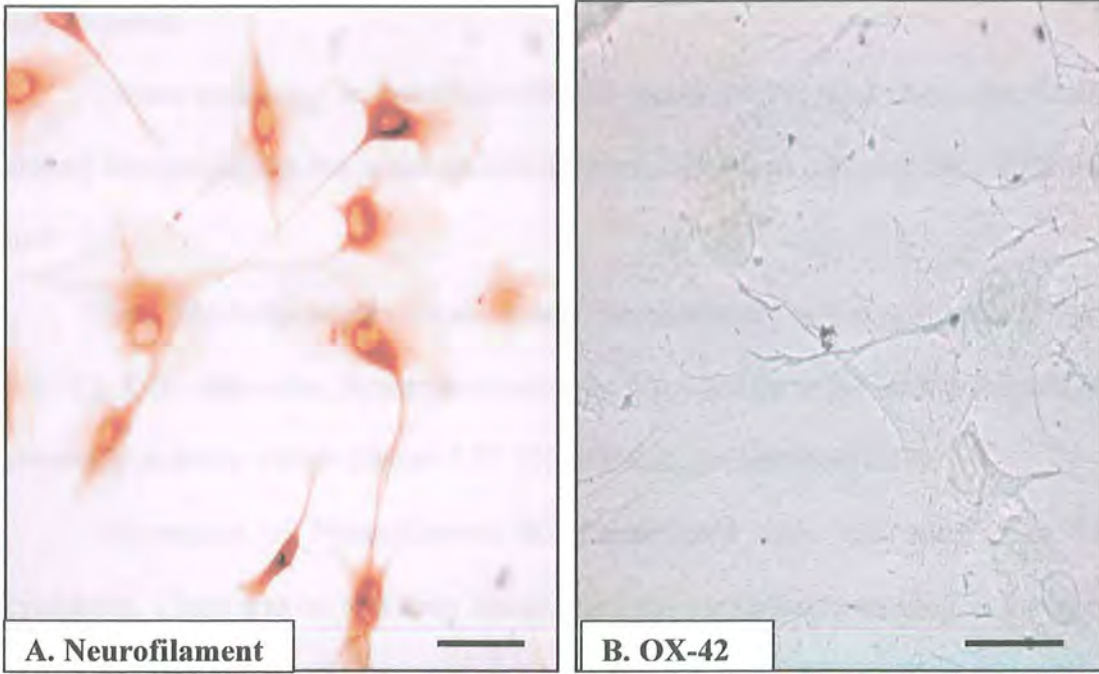


Figure 5.24. Expression of (A) neuronal marker Neurofilament and (B) dendritic marker OX-42 in untransfected BMSSCs. Neurofilament is highly expressed and present only in the cytoplasm. OX-42 was not expressed. Scale bar 10µm.

Rat BMSSCs do not express *Pax7* (Figure 5.22 A). NeuN was found expressed in the nucleus of BMSSCs but not in the cytoplasm as expected (Figure 5.22 B). The nuclear expression of NeuN in these stem cells was a surprise. NeuN expression was confined to very dark staining regions in the nucleus, possibly at a site of active chromatin, indicative of basal level expression of NeuN. NeuN is usually only expressed when a cell exits from neural progenitor cell status and becomes a fully differentiated neuron (Sarnat et al., 1998).

GFAP was expressed particularly in the cytoplasm but not in the nucleus of untransfected BMSSC cells (Figure 5.23 A). The staining was evenly spread within the cytoplasm.

Nestin expression in untransfected cells was in the cytoplasm and specifically around the nucleus but not in the nucleus (Figure 5.23 B), as expected for a stem cell line.

Beta III tubulin was expressed evenly throughout the cell at low levels (Figure 5.23 C). Ki67 expression in untransfected cells was specific to the nucleus, with dark chromatin staining visible (Figure 5.23 D), indicating proliferating cells.

Expression of Neurofilament in untransfected cells was specific to the cytoplasm. There was no cell body staining and the cytoplasmic staining was evenly distributed through out the cytoplasm (Figure 5.24 A). By contrast OX-42 expression was not detected in untransfected cells at all (Figure 5.24 B).

Thus BMSSCs express neural genes at low levels, thus indicating the potential of BMSSCs to differentiate along a neurogenic lineage.

## **CHAPTER 6**

### **DISCUSSION**

### 6.1 Justification for the use of Pax7d

In this study, the transcription factor *Pax7* was used to induce the differentiation of stem cells into a homogenous population of neurons. As a transcription factor, *Pax7* regulates the expression of downstream target genes that determine cell lineage and differentiation. *In vivo* *Pax7* has been implicated in cell specification of midbrain neurons during embryonic development (Basch et al., 2006). Given the importance of *Pax7* in CNS development we anticipated that by transfecting undifferentiated cells with *Pax7*, the cells will be directed along a neurogenic lineage.

The *Pax7* gene encodes several transcripts and each encoded isoform is believed to have a specific set of target genes, however this remains to be determined (White and Ziman, 2006). In this project the *Pax7d* transcript (Appendix 2) was used in transfections of both P19 and NIH3T3 cells.

Although no specific transcript of *Pax7* has been assigned an individually distinct function, *in vitro* experiments suggest that *Pax7d* may be important in neural differentiation. It has been demonstrated that the transcripts *Pax7b* and *Pax7d* are specifically expressed in the brain of adult mice (Ziman et al., 1997). Moreover when P19 cells were transfected with *Pax7d* the cells were induced to differentiate along a neurogenic lineage (Ziman et al., 2001). It is possible that the *Pax7d* transcript functions to regulate neural genes in the tectum to specify tectal neural fate (Thomas et al., 2004; Ziman et al., 2001).

Previously, transfection of P19 cells with either *Pax7* or *Pax6* resulted in neural differentiation of the cells. While it was determined that it was the expression of *Pax* genes that induced the neural differentiation of the cells, it was thought that possibly the default pathway of P19 cells may be neural. Therefore we assessed the



neural differentiation of several cell lines following transfection with *Pax7*. Our results showed that both gene and protein expression patterns were similar in all the cell lines assessed. Thus ectopic expression of *Pax7* has the ability to induce neural differentiation in multiple progenitor cell lines.

Expression of *Pax7* in multiple transfected cells induced upregulation of neural genes, indicating that upregulation of neural genes was due to *Pax7*. Stem cells were transfected with *Pax7* to assess the ability of this gene to induced neural differentiation for future transplant therapies. Experiments were conducted in P19 cells, NIH3T3 cells and BMSSCs to compare neural induction in all three cell lines. Sadly, while the transfection of BMSSCs was successful, the cells did not survive selection and no further results could be obtained. Future studies will concentrate on perfecting cell culture and transfection of these cells for future stem cell therapies

## **6.2 Novel findings**

When P19 stem cells were transfected with the gene *Pax7*, the expression levels of several genes were altered. The differences in the gene expression and protein levels were due to the presence of *Pax7*, since the control cells had little or no *Pax7* expression. It was clear from our results that neural differentiation genes were upregulated while glial specific genes were down regulated by *Pax7*.

Neurofilament is a structural protein present in neurons and also serves as a marker for neural differentiation; increased expression is an indication that the cells are moving towards neural differentiation. Neurofilament was upregulated in the presence of *Pax7* in transfected cells relative to untransfected cells. Stem cells would have little or no neurofilament expression, when assessed by immunocytochemistry and only low levels of neurofilament (basal level expression) were detected by RT-

PCR in untransfected cells. Both cell lines expressed neurofilament at a higher level after transfection with *Pax7*, indicating some differentiation towards a neuronal lineage.

Further support for this was the upregulation of NCAM in transfected cells. Neural cell adhesion molecule is a marker of mature migrating neurons. NCAM plays a vital role in neural cell proliferation and migration during development. It has important implications in synapse formation between neurons and the establishment of new connections through cell-cell adhesion. Spatial and temporal expression plays a pivotal role in neurogenesis during development (Ronn et al., 2000). A long chain of sialic acid is added to NCAM to form polysialic acid NCAM which is negatively charged (Kiss and Rougon, 1997). Neural migration is facilitated by the negative charge due to attachment of polysialic acid on NCAM which is a membrane spanning protein expressed on the neurons to help axonal growth and neural migration. Attachment of polysialic acid on NCAM prevents any synapse formation and promotes neural migration. NCAM is therefore an important cell membrane protein expressed by neurons, in which conjunction with polysialic acid aids neural migration and synaptogenesis. Our findings indicate NCAM was not expressed at all in the control untransfected cells but was expressed in transfected cells. NCAM expression in transfected cells suggested neural differentiation had been induced by the presence of *Pax7*.

Further experiments are required to determine whether increased *Pax7* expression in transfected cells, causes increased cell migration due to increased polysialic acid NCAM expression.

Beta III tubulin is also a structural protein found in neurons. Although tubulin is expressed widely in a variety of cells, the beta three isoform is particularly

expressed in neurons. In our experiments conducted here, as found previously (Blondheim et al., 2006) expression levels in untransfected cells were low. With introduction of *Pax7*, Beta III tubulin expression was increased.

Beta III tubulin forms the cytoskeleton structure of the neuron. Studies have shown that increased expression of Beta III tubulin in neural precursor cells indicates the formation of neuronal axons (Moskowitz and Oblinger, 1995). Our results are consistent with the finding by Lee and Pixley; who showed that Beta III tubulin is particularly expressed in high levels within the somata of new born neurons but shows little expression in mature neurons (Lee et al., 1990). Thus Beta III tubulin is implicated in building up of the cytoskeletal structure of differentiating stem cells towards a neural precursor lineage. Upregulation of Beta III tubulin in *Pax7*<sup>+</sup> stem cells may indicate that *Pax7* is a suitable transcription factor for use in differentiation of stem cells for transplant therapies. Untransfected BMSSCs however also expressed Beta III tubulin. Blondheim (Blondheim et al., 2006) who showed similar Beta III tubulin expression in untransfected BMSSCs and hypothesised that expression of low levels of neural markers in stem cells indicated their ability to differentiate along a neurogenic lineage.

Stronger evidence that *Pax7* induces neurogenic differentiation in stem cells is provided by the finding that NeuN was upregulated in transfected cells. NeuN is a nuclear protein antigen that is used to identify post mitotic neuron (Weyer and Schilling, 2003). It is used as a marker for neuronal maturation in the nervous system and is predominantly localised to the cell nucleus. Both P19 and NIH3T3 transfected cells expressed similar patterns of NeuN, indicating that *Pax7* upregulated NeuN and induced neural differentiation. NeuN has specific staining patterns; it is preferentially

found in areas of low chromatin density and virtually excluded from densely packed DNA (Lind et al., 2005).

GFAP has been used as a biomarker for neuroglial cells, as it is a protein usually expressed by astrocytes (Hausmann et al., 2000; Maubach et al., 2006). Of particular note was our finding that the expression level of GFAP was dramatically altered in the presence of the Pax7 protein when assessed by RT-PCR. Thus, introducing *Pax7* represses the expression of GFAP which possibly indicates repression of an astrocytic lineage (Lee et al., 2006), in transfected differentiating cells.

The protein level of GFAP appeared unchanged after *Pax7* transfection, in both P19 and NIH3T3 cells, which could be an indication that the turnover rate of GFAP is slow. The continuing protein expression of GFAP may be an indication that ubiquitin mediated protein degradation is active at very low levels for GFAP protein. Other studies have shown that different cellular proteins have variable turnover rates through ubiquitin mediated protein degradation (Ciechanover, 1991).

The upregulation of *Nestin* mRNA by *Pax7* transfection in P19 cells was an unexpected result. Nestin is an intermediate filament protein that has typically been associated with neuroepithelial cells and is an indicator of cells at an early stage of neural differentiation (Dahlstrand et al., 1995). During the development of CNS, proliferating neuroepithelial cells express *Nestin* (Lendahl et al., 1990). However, nestin is no longer considered to be exclusive to neural stem cells but rather is an indication of a multi potent progenitor cell, as it has also been shown to be expressed in muscle progenitor cells and some epithelial cells (Mokry and Nemecek, 1998). Since nestin expression in stem cells bridges the gap between neural progenitor stem cells and differentiated neurons, *Pax7* transfected cells expressing Nestin could be

said to be on a path towards neural differentiation. BMSSCs also expressed nestin, which indicates the potential of BMSSCs to differentiate along a neural lineage. Other studies have shown similar expression of Nestin in BMSSCs (Scintu et al., 2006; Tondreau et al., 2004).

The continued expression of *Nestin* in Pax7 transfected cells suggested that even though the cells expressed NeuN and other markers of mature neurons, the cells were still at an early stage of neural differentiation. Alternatively, Nestin expression after transfection could be an artefact of culture conditions. This is supported by a study that showed that the passage number and presence of serum in culture were two factors that affect expression of nestin (Wislet-Gendebien et al., 2003). Further work needs to be done to elucidate the reasons for the continued expression of nestin in Pax7 transfected cells.

Markers of specific dorsal midbrain neurons were consistently increased in Pax7 transfected cells. Ephrin A2 (Ephrin Alpha receptor 2) is a marker of mature differentiated neurons in the dorsal midbrain or tectum. Class A Ephrins are protein coupled receptors that are linked to the cell membrane by a GPI anchor. Ephrin molecules play a vital role as regulators of developmental processes such as axon guidance, cell migration and synapse formation (Wilkinson, 2001; Holmberg et al., 2005). These molecules provide a feedback system for cells at different stages of maturation and connectivity, and regulate the number of cells generated in the brain. Upregulation of Ephrin A2 in Pax7 transfected cells both P19 and NIH3T3 cells, provided further evidence that transfected cells were undergoing neuronal differentiation. While *Ephrin A2* might not be directly bound by the Pax7 transcription factor, it is certain that its expression is regulated by Pax7. This is supported by previous studies, which showed that Pax7 regulated expression of

*Ephrin A2* *in vitro* (Thomas et al., 2004) and *in vivo* (Thompson et al, 2006). *Pax7* and *ephrin A2* may regulate polarity and topography of the dorsal midbrain. Our findings here lend further support to these previous studies and indicated that transfection of stem cells with *Pax7* produced a specific midbrain neural progenitor cell, useful for future stem cell transplant therapies.

Engrailed is also a transcription factor that determines the structure and polarity of the dorsal midbrain (Itasaki et al., 1991). Expression of Engrailed was dramatically increased in transfected cells. Up regulation of Engrailed due to the presence of *Pax7* provided further evidence that *Pax7* induced differentiation of stem cells towards a neural lineage specific to the midbrain.

So far we have shown by RT-PCR and immunocytochemistry that *Pax7* upregulated key neuronal genes Neurofilament, Engrailed, NCAM, Beta III tubulin and down regulated astrocytic gene GFAP. The expression profile of these genes indicated that the transfected cells had differentiated into early stages neurons and not astrocytes. Increased expression of *Ephrin A2* which is a marker for tectal neurons and NeuN, a mature neuron marker as well as Engrailed provided further evidence that the differentiated cells were neurons specific to the tectum. Thus *Pax7* induced neurons may ultimately be used as cellular material for transplant therapies in the tectum.

It is clear from our results that while *Pax7* transfected cells expressed markers of differentiated neurons, they also expressed stem cell markers such as CD 90, CD44 and CD11B. Transfected cells also expressed Ki67, which is a marker of proliferation. NIH3T3 cells in particular expressed Ki67 after transfection with *Pax7*, when growth factors were withdrawn, the expression of proliferation markers

decreased. Growth factors are known to cause rapid proliferation in stem cells (Villa et al., 2000). Thus it was clear that transfected cells did not fully differentiate.

It is also now clear that production of a homogeneous population of neurons is not possible with the use of just one transcription factor. While the cells transfected with *Pax7* showed several characteristics of midbrain neurons, they still retained stem cell features. This may be an artefact of the stem cell lines used as they are engineered to remain proliferative. Alternatively, differentiation even *in vivo* may proceed via several steps in which markers of both differentiated and undifferentiated cells are present.

BMSSCs are suitable cellular material for transplant therapies, but they are difficult to culture. The cells proliferated slowly and when transfected remained firmly attached to the plastic, and thus were not transferable into immunocytochemistry wells. BMSSCs are also affected by small variations in culture conditions i.e. different serum batches affect growth conditions. Future research will need to take these factors into consideration to ensure optimal culture conditions of BMSSCs neural induction.

In summary, in *Pax7* transfected cells, neural genes were upregulated and cells differentiated towards a neural lineage. The key genes upregulated by transfection of *Pax7* were Neurofilament, Beta III tubulin, Engrailed 2, Ephrin A2, NCAM and NeuN. The cell types produced in two transfected cell lines were similar strengthening the case for the use of developmental transcription factor to produce specific neurons in culture.

### 6.3 Future directions

Further work is needed to characterise and carefully quantify which genes are expressed at a basal level in stem cells. Micro-array is a useful tool to assess gene expression at a basal level. The transfected cells also need further characterisation before use in cell replacement therapy. Transfection into freshly isolated BMSSCs may produce more consistent results.

Additional work is also needed to identify the ability of individual *Pax7* transcripts to regulate key genes associated with neural cell differentiation. Here, we only transfected one transcript, *Pax7d*; two or more transcripts may have yielded better results.

Research is currently underway to assess the key genes that are regulated by the transcription factor *Pax7* *in vivo*. This information may be useful in assessing the molecular mechanisms responsible for cell differentiation towards one lineage and not another.

Developmental genes are still thought to provide the best possible mechanism of producing differentiated neurons *in vitro* for use in stem cell transplant therapies. Our results provide further support for this theory.



## **CHAPTER 7**

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## APPENDIX 1

Vector sequence of pcDNA3.1D/V5-His-TOPO vector (Invitrogen)

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGC  
CGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCG  
AGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTT  
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## APPENDIX 2

The cDNA sequence of *Pax7d* transcript which was cloned into the vector with altered nucleotide sequence.

```
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```

Notes: [CAG] is included in the transcript *Pax7d* while [GTTTAG] is excluded from *Pax7*.

### APPENDIX 3

#### Buffers and solutions

##### **1. 10x Phosphate Buffered Saline (10x PBS)**

- Sodium chloride NaCl.....85g
- Di-hydrogen sodium phosphate.....4g
- Di-sodium hydrogen phosphate.....10.6g
- ddH<sub>2</sub>O.....1L
- pH7.4 with cHCl

##### **2. 10x Tris Buffered Saline (10x TBS)**

- TRIS base.....48g
- Sodium chloride.....36g
- ddH<sub>2</sub>O.....1L
- pH7.4 with cHCl

##### **3. 10x Tris Non-Saline (10x TNS)**

- TRIS base.....60g
- ddH<sub>2</sub>O.....1L
- pH7.4 with cHCl

##### **4. 4% Paraformaldehyde (4% PFA)**

- Paraformaldehyde.....40g
- Heat to dissolve PFA
- pH7.4 with orthophosphoric acid